A NOVEL ROLE FOR BONE MARROW-DERIVED CIRCULATING CELLS IN THE REPAIR OF INTESTINAL INFLAMMATION IN A MURINE PARABIOSIS MODEL

By

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore Maryland

July 2018

ABSTRACT

Mucosal repair after intestinal injury is thought to occur by a coordinated process combining proliferation and differentiation of intestinal stem cells with migration of healthy cells adjacent to the injury site. While this process is widely accepted, several lines of evidence suggest that other stem cell populations may contribute to mucosal healing in other organs, supported by the fact that bone marrow-derived cells contribute to the homeostasis of peripheral organs by their incorporation into heart, lungs, and liver to support physiological repair. We hypothesize that circulating bone marrow-derived cells promote healing of the injured mouse intestine, and tested this hypothesis in two injury models, namely radiation-induced enteritis and chemically-induced colitis. Using a parabiosis system in which two mice are surgically conjoined to develop a common circulation, we now show that bone marrow cells provide significant protection against intestinal damage caused by either ionizing radiation or chemical trinitrobenzenesulfonic acid (TNBS) in a parabiosis system. When bone marrow cells are depleted from donor partners in the parabiosis system by ionizing radiation, the rescue is lost, confirming the role of bone marrow cells in mediating the protective effects. Using tdTomato reporter mice, we determine that non-hematopoietic lineage cells migrate to the intestinal injury sites via the peripheral circulation in bone marrow cell transplant and a parabiosis system. The recruited bone marrow-derived cells are observed in the lamina propria, with little evidence of incorporation into the damaged epithelia. Furthermore, we show that bone marrow-derived cells localize differently within stroma as bone marrow-derived cells in the injured intestine congregate near the crypts while bone marrow-derived cells in the

uninjured intestine are spread throughout the villi and submucosa. These findings suggest that homing signals from the injured crypts may recruit bone marrow-derived cells to promote a supportive stem cell microenvironment during intestinal regeneration, and reveal a novel repair mechanism for intestinal injury, which may be therapeutically useful to mobilize a patient's bone marrow cells to aid intestinal repair.

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Preface

Acknowledgements

I would to express the deepest appreciation to mentor Dr. David Hackam for providing me with the extensive resources, professional lab environment, and in-depth training for me to make the transition from a student into a research scientist. I am grateful for Dr. Hackam's generous guidance and support, which made it possible for me to complete the thesis dissertation successfully.

I would like to thank all the key members of the Hackam lab who helped me to learn how to conduct the scientific research including Dr. Chhinder Sodhi, Dr. Hongpeng Jia, Dr. Peng Lu, Dr. Quinjie Zhou, Dr. Adam Werts, William Fulton, Thomas Prindle, Sanxia Wan, Emilyn Banfield, Dr. Mitchell Ladd, and Dr. Yukihiro Yamaguchi. Without their support, this dissertation would not have been possible.

I would also like to thank my thesis committee members including Dr. Dave Valle, Dr. Susan Michaelis, Dr. Dan Warren, and Dr. Linzhao Cheng for serving on my committee and taking time to provide the continued mentorship and advice throughout my Ph.D. training.

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INTRODUCTION

Healing from mucosal injury is of fundamental importance to gastrointestinal homeostasis [1]. Current dogma indicates that mucosal healing occurs through two parallel processes, namely *restitution* which involves the migration of healthy cells adjacent to the site of epithelial disruption[2], and *proliferation* which involves the generation of new epithelial cells from progenitor cells located within the crypts of Lieberkuhn[3].

Interestingly, clinical observations raise the possibility that these processes may be inadequate to account for the mucosal healing that occurs in certain clinical settings, and suggest that additional, unexplored healing processes may play a role. For instance, in the setting of radiation injury to the intestine, a loss of intestinal stem cells occurs as a direct result of radiation exposure, yet the intestinal mucosa still recovers[4], despite the apparent total destruction of the intestinal stem cells in the injured intestine[5 6]. Further, in the setting of advanced ileitis and colitis, marked denudation of the mucosal epithelia layer occurs, yet complete epithelial healing is still achieved even though the adjacent epithelial cells are widely and completely disrupted[7]. Such observations raise the possibility that previously unrecognized pathways towards mucosal healing may be involved and could therefore play important roles in the regulation of healing after mucosal injury[8 9 10 11]. In this regard, previous authors have suggested the possibility that circulating cells could migrate to sites of injury and contribute – either directly or through the paracrine release of molecules – to the healing response. In support of this possibility, stem/progenitor cells with mesenchymal stem cell or hematopoietic stem cell properties have been identified circulating in blood after injury[12 13], suggesting that the bone

marrow may serve as a reservoir for such cells. Moreover, previous authors have shown the incorporation of bone marrow-derived cells into the injured intestine[14], although such findings having been controversial[15 16], in part due to the lack of appropriate animal models used[17], and a reliance upon bone marrow-derived cell transplantation protocols which do not allow for the assessment of a stable bone marrow cell population that has developed and matured under physiologic conditions or in unperturbed states[18]. The potential role therefore of circulating stem cells in the healing of significant mucosal injury thus remains an open question.

To address this controversy, we have now employed a murine parabiosis system and established a shared circulation between surgically paired mice who were then subjected to either radiation-induced enteritis or chemically-induced colitis to mimic the clinical scenarios encountered above[19 20]. This approach allows us to develop a platform to investigate whether circulating stem/progenitor cells can participate in the repair of injured intestine, and to test the hypothesis that circulating cells originated from bone marrow participate in healing after intestinal injury. We now report that circulating cells migrate to the sites of injury, are incorporated into the injured mucosa, and play a critical and previously unrecognized role in healing after mucosal injury in two complementary models.

SPECIFIC THESIS AIMS

Aim 1: To assess intestinal healing using parabiosis mice vs. non-parabiosis mice in different injury models.

Aim 2: To define the repair mechanisms of circulating stem cells in injured intestine.

Aim 3: To characterize the migrated cell types promoting intestinal tissue repair.

Aim 4: To investigate the functional consequences of pro-healing agents within the circulation and their depletion in intestinal regeneration.

Aim 1: To assess intestinal healing using parabiosis mice vs. non-parabiosis mice in different injury models.

AIM 1 INTRODUCTION

The intestines are composed of small and large intestine. The main role of this 750 cm long and tube shaped organ is the absorption of water and nutrients. The small intestine is the longest organ in human body and divided into three segments—the duodenum, jejunum, and ileum. Villi, small finger-like structures in small intestine, maximize the absorption of most of nutrients and minerals from food. The large intestine is wider in diameter compare to the small intestine and divided into cecum, colon, and rectum. The colon consolidates stool by absorbing water from wastes. Abundant goblet cells, which secrete mucins, optimize the passage of feces through relatively flat epithelium.

Healing from mucosal injury is of fundamental importance to gastrointestinal homeostasis. Current dogma suggests that mucosal healing occurs through two parallel processes, which include *restitution* i.e. the migration of healthy cells adjacent to the site of epithelial disruption towards to epithelial defect, and *proliferation* i.e. the generation of new epithelial cells from progenitor cells located within the crypts of Lieberkuhn. Importantly, clinical observations suggest that these two processes may be inadequate to account for the mucosal healing that occurs in the setting of severe mucosal injury, implying that additional, unexplored healing processes may play a role.

Injury to the intestine can be caused by various factors, including infections, radiation damage secondary to management of cancers, and inflammatory bowel diseases (IBD). While intestinal inflammation is a common cause of mortality[21], there is a substantial unmet need in the effective treatment of intestinal diseases[22 23].

The small intestine is one of the most radiosensitive organs due to the rapid rate of intestinal stem cells (ISC) division. Gamma irradiation induces DNA damage and apoptosis of ISC, subsequently leading to the destruction of intestinal crypts and mucosal lining. Unlike the single-strand break damage, double-strand breaks of DNAs by gamma irradiation are more likely to be incorrectly repaired, leading to genomic instability. If the double-strand breaks of DNAs are unrepairable, the cells engage various damage sensing mechanisms, resulting in apoptosis. Additionally, the increased production of reactive oxygen species in ISC by ionizing radiation causes mutagenesis, inhibition of covalent modifications of lipids and proteins, and carcinogenesis.

While a high dose of gamma radiation completely destroys some crypts, the surviving crypt stem cells can go through cell division to increase their numbers to maintain epithelial homeostasis, restoring sufficient numbers of crypts after intestinal injury. However, it is unclear whether this restoration process requires activation of a quiescent stem cell population within the intestinal structure or involves replenishment cells from the exogenous source such as the bone marrow.

Radiation enteritis is caused by the often unavoidable exposure of the small intestine to radiation during oncological treatment of other organs[24]. The gastrointestinal injury secondary to radiotherapy occurs in many patients undergoing abdominal and

pelvic radiotherapy[24 25]. Symptoms of radiation enteritis include weight loss, rectal bleeding, diarrhea, and vomiting. While the number of cancer patients is expected to increase to around 20 million by 2030 and sixty percent of oncology patients are expected to receive radiation therapy, there are no effective treatments available[26]. However, in some cases of radiation injury to the intestine, a loss of intestinal stem cells occurs due to gamma radiation induced-DNA breakages, yet the intestinal mucosa still recovers.

IBD, which includes Crohn's disease and ulcerative colitis, are characterized as chronic inflammatory status of intestinal tract. While as many as 1.6 million Americans suffer from IBD, current therapeutic approaches have been often ineffective. Patients manifest varying degrees of symptoms and complications—diarrhea, weight loss, bloody stool, perforated colon, and bowel obstruction. While symptoms may range from mild to severe, the life-long medical condition for which no successful therapies are developed can adversely affect patient's quality of life. With the current pharmacologic options, only 50% of patients respond to therapy. Additionally, the efficacy of biologic drugs declines over time due to patients' development of antibody against the drugs[27]. What interests us is that, in the setting of advanced inflammatory bowel disease, marked denudation of the mucosal epithelium occurs, yet epithelial healing is still achieved, even though the adjacent epithelial cells may be completely lost. Such observations raise the possibility that previously unrecognized pathways could play key roles in intestinal mucosal healing after mucosal injury, especially under conditions in which proliferation and restitution are unlikely to be effective.

In this regard, previous authors have suggested the possibility that circulating, extra-intestinal cells can migrate to sites of intestinal injury where they may contribute – either directly or in a paracrine fashion – to the mucosal healing response. Recent studies provide evidence that bone marrow-derived circulating cells are recruited to the different organs in response to injury and engraft in non-hematopoietic tissues[8 28 29 30]. While there are several lines of evidence showing that possible incorporation of migrated bone marrow-derived cells into injured intestine[31], little is known about its functional relevance or specific repair mechanisms. Some of the uncertainty stems from the fact that many studies rely on bone marrow-derived cell transplantation protocols. For bone marrow-born cells to participate in tissue repair in distal organs, those cells need to egress from marrow, enter into the peripheral circulation, and migrate to the injury sites. However, the transplantation approaches do not allow assessment of stable bone marrow cell population that have matured under physiological conditions.

My thesis work employs a murine parabiosis system to discover the role of humoral factors in intestinal regeneration and repair. Parabiosis mice are constructed by surgically conjoining two animals together, leading to the development of a single, shared circulatory system[32]. Parabiosis has been used to investigate the systemic effects of circulating factors for the last 150 years. Since it was first introduced by Dr. Bert in the 19th century, utilization of this technique led to the discovery of leptin, the satiety factor, which resulted in a Nobel prize for Dr. Jeffrey Friedman. Parabiosis is a powerful experimental approach to study stem cell biology and regeneration research, and allows

us to investigate the biological effects of circulating humoral factors that originate from the conjoined partner[33].

The extensive shared circulatory system between two animals in parabiosis provides a perfect platform to examine whether bone marrow-derived cells can exit the marrow, circulate in peripheral blood, and migrate to intestinal inflammation sites under the pathophysiological conditions[19].

While there are no animal models that completely recapitulate all of the features of a multifactorial disease such as IBD, well-established experimental models using dextran sulfate sodium (DSS) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) allow us to induce IBD in mice and analyze the disease and repair mechanisms. In our study, intrarectal instillation of TNBS was preferred due to feasibility and practicality issues associated with feeding of DSS solution to only one parabiont in a parabiosis system. In the TNBS injury model, TNBS is dissolved in 50% ethanol and instilled directly into colon to induce acute colitis. Ethanol breaks down the mucosal barrier, which promotes the transmural absorption of TNBS in colon. TNBS is believed to haptenize the self-antigens to T cells, eliciting an intense immune response.

In the current study, we utilize a murine parabiosis system in two different injury models– radiation-induced enteritis and chemically-induced colitis–, which allow us to investigate whether circulating stem/progenitor cells can participate in the repair of injured intestine. We suggest that comparison of gut injuries which are caused in different organs— small intestine vs. large intestine— and by different mechanisms— intestinal

stem cells vs. epithelial cells— in parabiont mice allows us to interrogate mucosal injury and repair at defined time points.

AIM 1 METHODS

Statement of ethics

All animal procedures were conducted in accordance with National Research Council's Guide to the Care and Use of Laboratory Animals, and all of the animal protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins university. All mice were housed under a controlled temperature (25 ℃) and light-dark cycle of 12 hours.

Parabiosis

Age matched, adult (3- to 12-months-old) female wild-type mice (C57BL/6 background) or transgenic tdTomato mice (C57BL/CAG-tdTomato) were used to generate parabiotic pairs in mice that had been co-housed for two weeks prior to surgery to promote harmonious cohabitation. For the parabiosis procedure, mice were anesthetized by intramuscular injection with 100 mg Ketamine/kg body weight and 15 mg Xylazine/kg body weight. A skin incision was made on the flank from the elbow to the knee joint. The tibia and ulna of each animal were sutured together with 4-0 biosyn (Covidien) to bind forelimbs and hindlimbs. The abdominal walls of each mouse were also sutured together using 5-0 monocryl (Ethicon) to promote the formation of

microvasculature between the two animals. The skin incisions on the flanks were then closed using 7 mm wound clips. Immediately following surgery, each parabiont was administered with sterile normal saline to prevent dehydration, Baytril to minimize infection, and Buprenorphine to manage pain.

Parabiotic cross circulation

After 4 weeks, the presence of a joint circulation was assessed by detection of tdTomato^{+} cells in blood and intestine from a wild-type parabiont, which was surgically connected to a tdTomato mouse. Parabiosis mice were anaesthetized by isoflurane inhalation and the blood was collected by retro-orbital bleeding. Peripheral blood smear slides were examined by fluorescence microscopy for presence of tdTomato^{+} cells in a wild-type parabiont. For flow cytometry analysis, red blood cells were removed by incubation in lysis buffer and washed with FACS buffer (0.5% BSA in PBS) three times. Remaining cells were then resuspended in FACS buffer. In a wild-type and tdTomato parabiosis pair, the composition of nucleated cells in peripheral blood was approximately 50% of tdTomato+ and 50% tdTomato-. BD Accuri C6 flow cytometer was used to collect flow cytometry data. Data analysis was performed using FlowJo software. Fluorescent microscopic observation of intestine from the wild-type parabiont demonstrated detectable tdTomato+ cells. Also, formation of blood vessels on the incision site was confirmed visually and the image was taken by iPhone SE's 12-megapixel, rear-facing camera.

Irradiation injury model and TNBS-induced colitis

One month after creation of parabionts, whole-body gamma irradiation was applied after mice were anesthetized by intramuscular injection with a solution consisting of 100 mg Ketamine/kg body weight and 15 mg Xylazine/kg body weight. A Gammacell 40 Exactor (a dual Caesium137, MDS Nordion) was used to perform whole body irradiation, where lead shielding was used to protect the non-irradiated parabiont pair as a control. Mice were exposed to 8 or 12gy irradiation and harvested after 3 days. In parallel studies, chemical colitis was induced according to the protocol of Wirtz S et al[34] in which mice were pre-sensitized by applying 1.5 mg of TNBS (Sigma-Aldrich, St. Louis, MO) to the skin. On day 8, mice that had been fasted at least 12 hours were anesthetized by isoflurane inhalation and then 2.5 mg of TNBS dissolved in 100 ul of 50% (v/v) ethanol solution was instilled rectally by using a 3.5 French catheter. Mice were kept in a vertical position with the head down for 3 min. Control mice were administered with 100 ul of PBS in a similar manner. Mice were checked daily with respect to their general condition, body weight, consistency of stools, and occult blood in stools. To identify proliferating cells, all mice were intraperitoneally injected with 100mg/kg of Bromodeoxyuridine (BrdU) 20 hours before euthanizing.

Harvest and processing of tissue specimens

At the end of the experimental period, small intestine or colon were harvested from the mice for longitudinal sections. About 1 cm of intestinal tissues were collected for longitudinal sections. Tissues were fixed overnight in 4% paraformaldehyde at 4℃,

and washed three times in PBS. For paraffin sections, tissues were dehydrated and embedded in paraffin, and then sectioned at 5 um thickness. For cryosections, fixed tissues were incubated for 48 hours in 30% sucrose in PBS at 4˚C and then embedded in optimal cutting temperature (OTC) medium (General Data). Sections were cut at 5-14 μm thickness.

Histology and histopathological scoring of tissue sections

The distal small intestine and colon were stained with hematoxylin and eosin (H & E). Histological damage scores of small intestine and colon tissues were graded (0=None, 1=Mild, 2=Moderate, 3=High) by a pathologist in a blind fashion based on the grading guidelines summarized in **Table 1-1**. Briefly, the following pathological criteria were used to assess the radiation enteritis—preservation of epithelial structure, glandular dropout, prominent Paneth cells, and infiltration of inflammatory cells in the lamina propria. In TBNS-induced colitis, colon was assessed based on epithelial injury, goblet cell phenotype, glandular dropout, inflammatory lamina propria expansion, ulceration, pseudomembrane, ischemic changes, and mucosal erosion. For goblet cell staining, paraffin sections were stained with Alcian blue solution and counterstained with nuclear fast red solution (Sigma-Aldrich, St. Louis, MO).

Immunohistochemical staining

Immunohistochemistry was performed on 5-14 μm paraffin or cryosections as follows: cryosections were thawed at room temperature and rehydrated with PBS. Paraffin sections were first warmed to 63 °C in a vacuum incubator (Isotemp Vacuum Oven, Fisher Scientific) then washed immediately twice in xylene, gradually re-dehydrated in ethanol (100%, 95%, 70%, water), and then processed for antigen retrieval in citrate buffer (10mM pH6.0)/microwave (1000 watt, power level 10, 6 minutes). For BrdU staining, samples were incubated in 2M hydrochloric acid (HCL) solution (40 min, room temperature) before the blocking step. Samples were then washed with PBS, blocked with 1% BSA/5% donkey-serum (1 hour, room temperature), then incubated overnight at 4°C with primary antibodies (1:200 dilutions in 0.5% BSA), washed 3 times with PBS, incubated with appropriate fluorescent labeled secondary antibodies (1:1000 dilution in 0.5% BSA, Life Technologies Inc). For apoptosis assays, samples were incubated with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection solution (In Situ Cell Death Detection Kit, TMR red, Roche) for 1hr at 37°C after secondary antibody incubation step. Slides were incubated with the nuclear marker DAPI (Biolegend), followed by multiple washes, and slides were then mounted using Gelvatol (Sigma-Aldrich) solution prior to imaging using a Nikon Eclipse Ti Confocal microscope under appropriate filter sets.

Quantitative real-time PCR

Total RNA was extracted from the small intestine or colon using RNeasy Mini Kit (Qiagen). 0.5 ug of each RNA sample were reverse transcribed into cDNA using the

QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed using iTaq UniverSYBR Green SMX 5000 (Biodrad) in the Bio-Rad CFX96 Real-Time System (Bio-Rad) with the primers listed in **Table 2-1** relative to the housekeeping gene ribosomal protein large, P0 (*RPL0*).

Statistical analysis

Data are shown as mean ± SEM and were analyzed using one-tailed Student's t tests for comparisons between two groups, one-way analysis of variance (ANOVA) with Sidak's test for multiple comparisons using PRISM version 7.0 (GraphPad). $p < 0.05$ was considered to be significant for all experiments.

AIM 1 RESULTS

A shared circulatory system is developed in parabiotic mice

To investigate the potential involvement of circulating cells or other factors in intestinal repair, we first sought to establish parabiotic mice by suturing the skin and muscle wall on the flank of two animals, and initially surgically joined a wild-type (C57BL/6 background) mouse to either another wild-type mouse or a transgenic ROSA26-CAG-tdTomato mouse, which expresses the red fluorescence reporter protein herein called TdTomato constitutively in all cells (Figure 1-1).

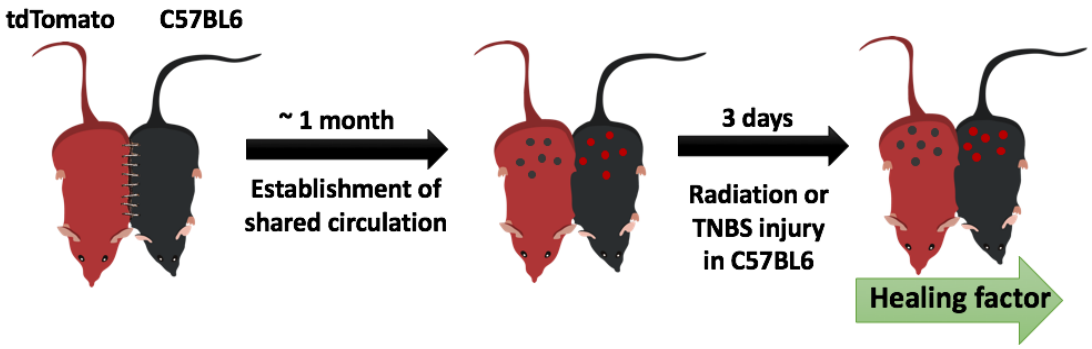


Figure 1-1. Diagram illustrating the murine parabiosis model to analyze the vascular cell fates of tdTomato$^+$ circulating cells in the injured partner. Intestinal injury was induced by whole-body irradiation or intrarectal TNBS instillation in the WT mouse that was surgically jointed to tdTomato mouse.

To evaluate the degree to which a shared circulation was obtained, we assessed the macroscopically appearance of microvasculature across the shared incision (Figure 1-2), and performed flow cytometric analysis of WT- tdTomato pairings, we determined that the WT mouse contained approximately 52% of tdTomato$^+$ nucleated blood cells in the circulation (Figure 1-2), which could be readily appreciated on the blood smear (Figure 1-2), and in the small intestine from the WT parabiont (Figure 1-2), thereby establishing that parabiosis was achieved.

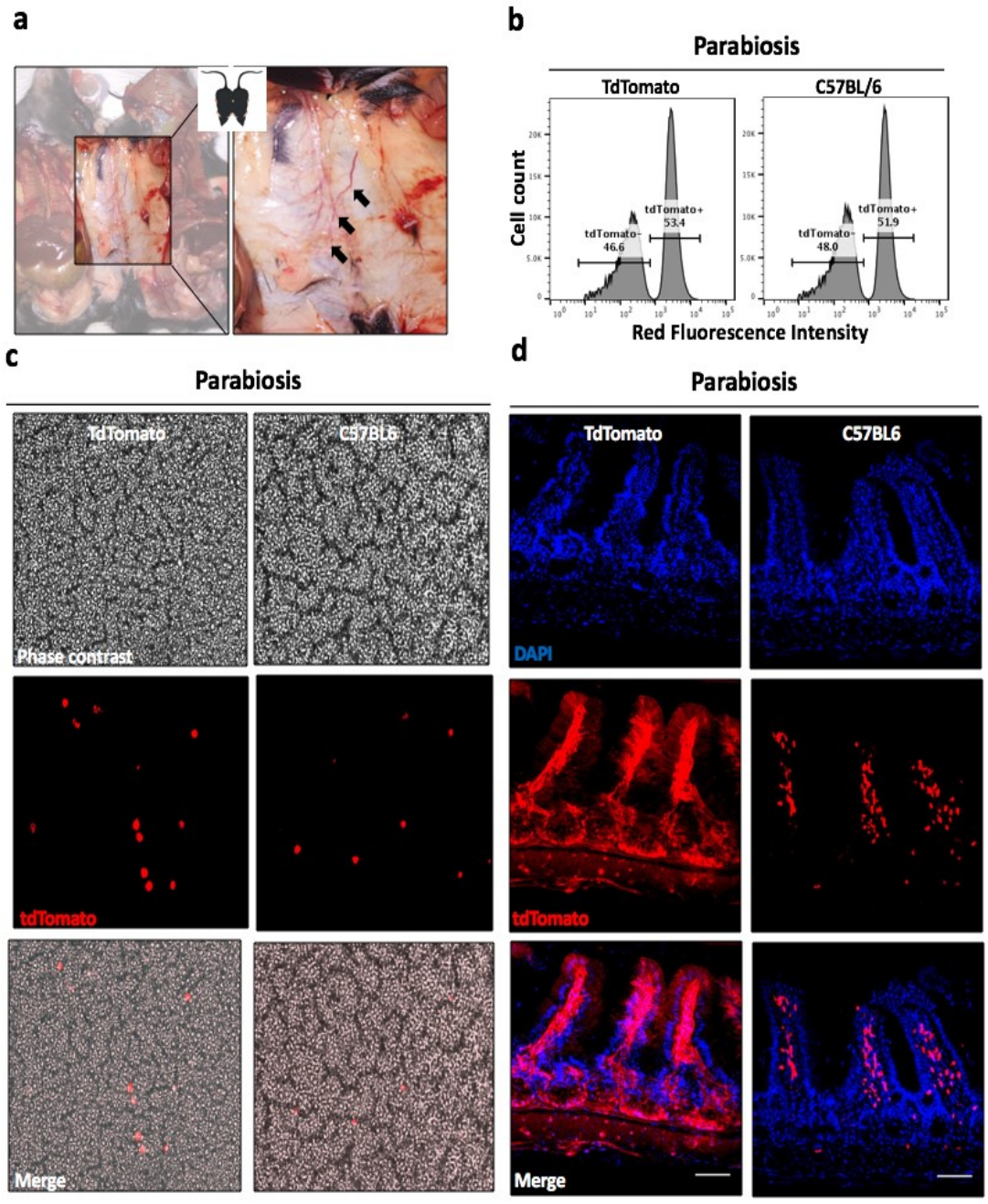


Figure 1-2. Proof of successful parabiosis system. (a) Shared circulatory system at the tissue level. Blood vessels (arrows) were developed across the suture site where

the skin of two animals was conjoined. (b) Flow cytometry analysis shows the approximately 50% of tdTomato$^+$ nucleated blood cells circulating in the peripheral blood of a WT parabiont (n=4 pairs), conjoined to tdTomato mouse. (c) Shared circulatory system in blood cells. In the parabiosis system of a tdTomato mouse with a WT mouse, the blood smear shows that the WT mouse gained tdTomato$^+$ blood cells. Original magnification 20x. (d) Shared circulatory system at the cellular level. In the parabiosis system of a tdTomato mouse conjoined with a WT mouse, partner-derived tdTomato$^+$ cells were observed in the small intestine of WT. Original magnification 20x; scale bars 100 µm.

Parabiosis provides protection from intestinal injury and promotes mucosal healing

We first assessed the effects of pairing on gastrointestinal mucosal injury in two complementary intestinal injury models, namely whole-body irradiation which targets intestinal stem cells (8-12 Gy) or intra-rectal administration of trinitrobenzenesulfonic acid (TNBS) which directly injures the intestinal epithelium (Figure 1-1). As shown in Figure 1-3, the body weight of 8 Gy-irradiated unpaired mice steadily declined to below 80% of the original body weight 12 days after injury, at which point mice were euthanized per protocol. Strikingly, the body weight of parabionts who were irradiated at the same dosage did not lose weight, and actually were found to be at the original body weight between days 12 and18, such that there was a significant body weight difference between unpaired and paired mice at days 2, 3, 5, 9 and 11 (Figure 1-3). Histological evaluation (Table 1-1) of the intestinal mucosa at day 3 after irradiation revealed

decreased numbers of crypts with atrophic villi accompanied by infiltration of inflammatory cells in the lamina propria in non-parabiosis mice, findings which were significantly reduced in the parabiotic mice and quantified in Figure 1-3. To evaluate whether the protection provided by a parabiotic pairing was specific to radiation enteritis, we next induced TNBS colitis to either paired or unpaired mice, and observed that while unpaired mice sustained significant weight loss which was most pronounced on day 3 after chemical instillation, parabiotic pairs showed dramatically reduced weight loss (Figure 1-3). Macroscopic changes such as shortened, thickened, and necrotic morphology of colon in TNBS-received unpaired mice (Figure 1-3) and microscopic changes including epithelial injury, crypt degeneration, inflammatory infiltrate into the lamina propria were noticeable at day 3 after TNBS injection in the unpaired mice yet all significantly reduced in parabionts (Figure 1-3). Taken together, these results suggest that the parabiosis system promotes restoration of the crypt and epithelium structure in intestine in both injury models, providing protection from intestinal inflammation.

a

Histological parameters for irradiated small intestine	Histology score for colitis
Preservation of epithelial architecture -Epithelial injury/distorted villi -Thinner villi -Shortened length of villi -Denuded villi	Epithelial injury (increased number of apoptotic bodies in crypts, crypt degeneration)
	Goblet cell phenotype (mucin depletion or mucin extrusion)
Decreased number of crypts -Glandular dropout	Decreased number of crypts (Glandular dropout)
	Inflammation (inflammatory lamina propria expansion)
	Ulceration
Prominent Paneth cells	Pseudomembrane
	Ischemic changes
Infiltration of inflammatory cells in the lamina propria (lamina propria expansion)	Mucosal erosion

0=None, 1=Mild, 2=Moderate, 3=High

Table 1-1. (a) Histological scoring system for radiated induced enteritis and TNBS-induced colitis.

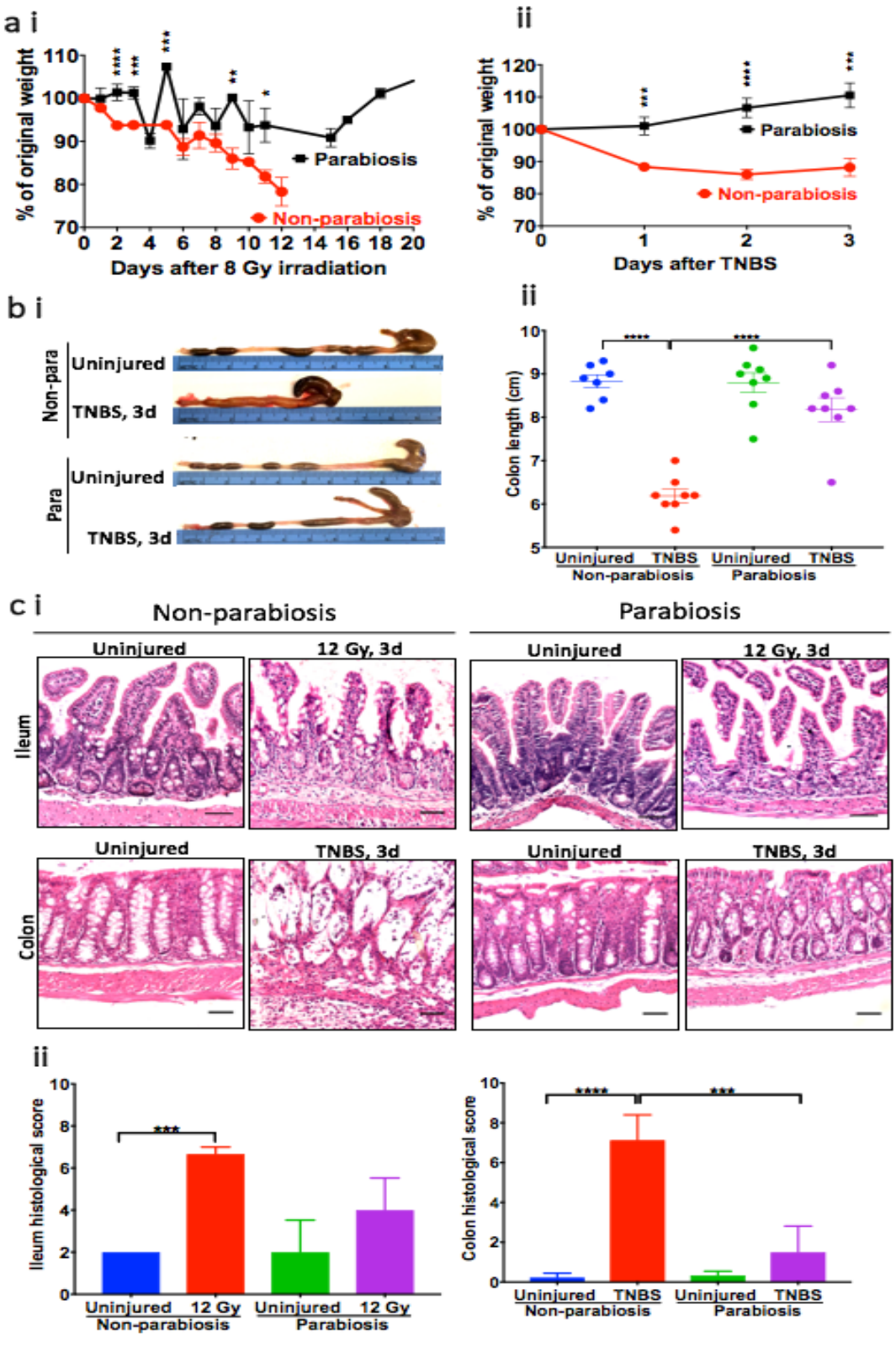

a i
% of original weight
Parabiosis
Non-parabiosis
Days after 8 Gy irradiation
ii
% of original weight
Parabiosis
Non-parabiosis
Days after TNBS
b i
Non-para
Uninjured
TNBS, 3d
Para
Uninjured
TNBS, 3d
ii
Colon length (cm)
Uninjured
TNBS
Uninjured
TNBS
Non-parabiosis
Parabiosis
c i
Non-parabiosis
Parabiosis
Uninjured
12 Gy, 3d
Uninjured
12 Gy, 3d
Ileum
Uninjured
TNBS, 3d
Uninjured
TNBS, 3d
Colon
ii
Ileum histological score
Uninjured
12 Gy
Uninjured
12 Gy
Non-parabiosis
Parabiosis
Colon histological score
Uninjured
TNBS
Uninjured
TNBS
Non-parabiosis
Parabiosis

Figure 1-3. Parabiosis mice are protected from radiation-induced enteritis and TNBS-induced colitis. (a) (i) Body weight change in an unpaired mouse (n=14) and parabiont mouse (n=7 pairs) after exposure of 8 Gy radiation. (ii) Body weight change in an unpaired mouse (n=13) and parabiont mouse (n=11 pairs) after TNBS instillation. Mice whose weight loss of 20% or more were euthanized. Data are presented as means ± SEM. (b) (i) Macroscopic images of colons from day3 after TNBS instillation. (ii) Colon length of TNBS-treated unpaired mice (n=7-8 as indicated) and parabiosis mice (n=8 pairs). Data are presented as means ± SEM. (c) (i) Representative H&E-stained sections from an unpaired mouse and parabiont mouse. Ileum 3 days after exposure of 12 Gy radiation (top); Colon 3 days after TNBS instillation (bottom). Original magnification 20x; scale bars 100 μm. (ii) Histological damage level was scored in a blind manner. Unpaired mouse (n=5-6) and parabiont mouse (n=3 pairs) from radiation injury model (left); Unpaired mouse (n=8-9) and parabiont mouse (n=6 pairs) from TNBS injury model (right). Data are presented as means ± SEM. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

Additional evidence that parabiosis reduced the degree of mucosal injury is shown in Figure 1-4 and Figure 1-5, indicating that while radiation and TNBS administration leads to an increase of pro-inflammatory cytokines and chemokines in the ileum or colon in unpaired mice, these were significantly reduced in the intestine of parabiotic pairs. Moreover, both radiation injury and TNBS colitis resulted in increased

crypt apoptosis in unpaired mice that was restored in the presence of parabiosis (Figure 1-4). It is noteworthy that the presence of parabiosis restored the effects of radiation injury and TNBS on crypt proliferation, which was reduced in radiated unpaired mice and increased in the presence of a parabiotic pair (Figure 1-4), while TNBS colitis was associated with crypt derangement and loss of goblet cells in the colon which was restored in the presence of a parabiotic pairing (Figure 1-5). Taken in aggregate, these findings indicate that the presence of a parabiotic partner reduces intestinal injury and/or promotes healing, and providing proof that circulating factors can be induced.

a

Gene	Forward primer	Reverse primer	Size
RPLO	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC	143 bp
TNF-α	TTCCGAATTCACTGGAGCCTCGAA	TGCACCTCAGGGAAGAATCTGGAA	144 bp
IL-1β	AGTGTGGATCCCAAGCAATACCCA	TGTCCTGACCACTGTTGTTTCCCA	175 bp
CCL2	ATGCAGTTAACGCCCCACTC	CCCATTCCTTCTTGGGGTCA	171 bp
SI	ATCCAGGTTCGAAGGAGAAGCACT	TTCGCTTGAATGCTGTGTGTTCCG	154 bp
CAR1	ACAGTAGCAACCAATCTGTTCTG	AGGCCATCAGCCTTGGAGA	207 bp
Lipocalin2	ACAACCAGTTCGCCATGGTAT	AAGCGGGTGAAACGTTCCTT	121 bp
MucII	TAGTGGAGATTGTGCCGCTGAAGT	AGAGCCCATCGAAGGTGACAAAGT	168 bp
iNOS	CTGCTGGTGGTGACAAGCACATTT	ATGTCATGAGCAAAGGCGCAGAAC	167 bp
Lgr5	TGAGCGGGACCTTGAAGATTTCCT	TACCAAATAGGTGCTCACAGGGCT	116 bp
Lysozyme	AAGCTGGCTGACTGGGTGTGTTTA	CACTGCAATTGATCCCACAGGCAT	178 bp
Dlck1	CAAATGCCGAGGCAAAGAGCACAT	ACAGTTCAGTCGGCACATCCATCT	113 bp
IL-6	CCAATTTCCAATGCTCTCCT	ACCACAGTGAGGAATGTCCA	182 bp
CXCL2	TCCAGAGCTTGAGTGTGACG	CTTCCGTTGAGGGACAGCAG	196 bp

Table 1-2. (a) List of primers used in real-time RT-PCR

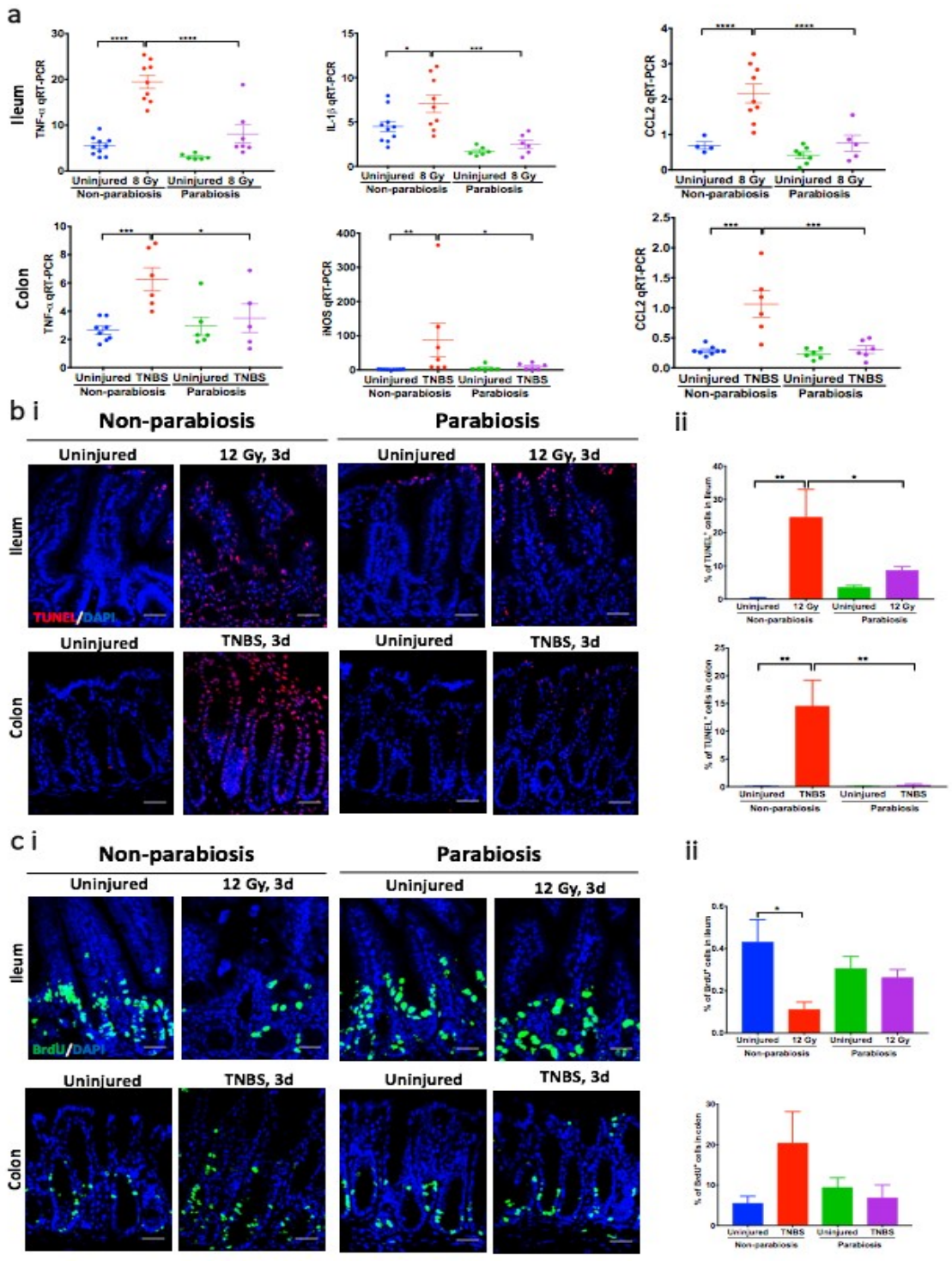

a
Ileum
TNF-α qRT-PCR
IL-1β qRT-PCR
CCL2 qRT-PCR
Colon
TNF-α qRT-PCR
iNOS qRT-PCR
CCL2 qRT-PCR
Uninjured 8 Gy
Uninjured TNBS
Non-parabiosis
Parabiosis
b i
Non-parabiosis
Parabiosis
Uninjured
12 Gy, 3d
TNBS, 3d
Ileum
Colon
TUNEL/DAPI
ii
% of TUNEL+ cells in Ileum
% of TUNEL+ cells in colon
12 Gy
c i
BrdU/DAPI
% of BrdU+ cells in Ileum
% of BrdU+ cells in colon

Figure 1-4. Cell Proliferation is increased and cell death and inflammation is decreased in parabiosis system. (a) RT-qPCR of cytokines and chemokine in non-parabiosis mouse and parabiont mouse. Ileum of unpaired mouse (n=9-10 as indicated) and parabiont mouse (n=7 pairs) from radiation injury model (top); Colon of unpaired mouse (n=6-9 as indicated) and parabiont mouse (n=7 pairs) from TNBS injury model (bottom). Relative mRNA levels which were normalized to that of RPLO are shown. Data are shown as Mean ± SEM. (b) (i) Immunohistochemistry of TUNEL from a non-parabiosis mouse and parabiont mouse. Ileum from radiation injury model (top); Colon from TNBS injury model (bottom). Original magnification 40x; scale bars 50 μm. (ii) Quantification of TUNEL fluorescence intensity normalized to DAPI per 40x FOV. Ileum of unpaired mouse (n=4-6 as indicated) and parabiont mouse (n=3 pairs) from radiation injury model (top); Colon of unpaired mouse (n=4-5 as indicated) and parabiont mouse (n=3 pairs) from TNBS injury model (bottom). Each value represents the average of technical replicates from one mouse. Results are presented as means ± SEM. (c) (i) Immunohistochemistry of BrdU from a non-parabiosis mouse and parabiont mouse. Ileum from radiation injury model (top); Colon from TNBS injury model (bottom). Original magnification 40x; scale bars 50 μm. (ii) Quantification of BrdU fluorescence intensity normalized to DAPI per 40x FOV. Ileum of unpaired mouse (n=4-6 as indicated) and parabiont mouse (n=3 pairs) from radiation injury model (top); Colon of unpaired mouse (n=4-5 as indicated) and parabiont mouse (n=3 pairs) from TNBS injury model (bottom). Each value represents the average of

technical replicates from one mouse. Results are presented as means ± SEM. (*p < 0.05, **p < 0.01, ***p < 0 . 001, ****p < 0.0001)

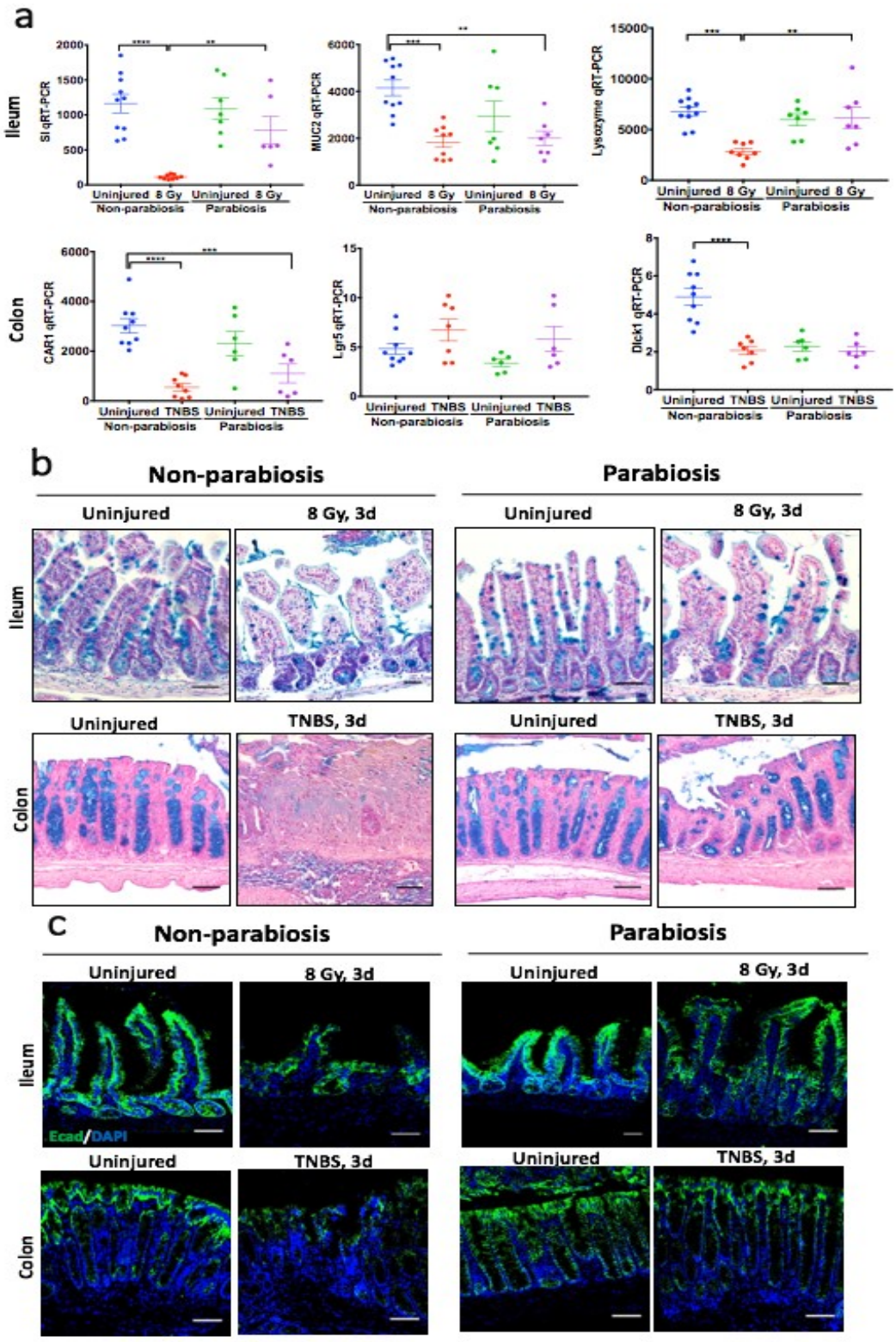

a
Ileum
SI qRT-PCR
MUC2 qRT-PCR
Lysozyme qRT-PCR
Colon
CAR1 qRT-PCR
Lgr5 qRT-PCR
Dick1 qRT-PCR
Uninjured
8 Gy
TNBS
Non-parabiosis
Parabiosis
b
Non-parabiosis
Parabiosis
Uninjured
8 Gy, 3d
TNBS, 3d
Ileum
Colon
C
Non-parabiosis
Parabiosis
Uninjured
8 Gy, 3d
TNBS, 3d
Ileum
Colon
Ecad/DAPI

Figure 1-5. Cell differentiation after injury is increased in parabiosis system. (a) RT-qPCR of intestinal cell markers in non-parabiosis mouse and parabiont mouse. Ileum of unpaired mouse (n=9-10 as indicated) and parabiont mouse (n=7 pairs) from radiation injury model (top); Colon of unpaired mouse (n=6-9 as indicated) and parabiont mouse (n=7 pairs) from TNBS injury model (bottom). Data are shown as Mean ± SEM. (b) Representative Alcian blue-stained sections from a non-parabiosis mouse and parabiont mouse. Ileum 3 days after exposure of 8 Gy radiation (top); Colon 3 days after TNBS instillation (bottom). Original magnification 20x; scale bars 100 μm. (c) Representative Ecad stained sections from a non-parabiosis mouse and parabiont mouse. Ileum 3 days after exposure of 8 Gy radiation (top); Colon 3 days after TNBS instillation (bottom). Original magnification 20x; scale bars 100 μm. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

AIM 1 DISCUSSION

The intestinal epithelium is one of the most rapidly renewing tissue, in which intestinal stem cells continuously replenish multiple intestinal cell types. While it is believed that resident stem cells play a critical role in replenishment of intestinal epithelium, interestingly, normal intestinal homeostasis is observed despite an apparent loss of Lgr5-expressing intestinal stem cells[4]. It was shown that circulating bone marrow-derived stem cells contribute to the mucosal healing in coeliac disease patients whose intestinal stem cells are depleted[13]. This remarkable regeneration response suggests the

possibility that other stem cells may coordinate the regeneration process. Several studies show that alternative intestinal progenitor cells or already differentiated intestinal cells can gain "stemness" and give rise to intestinal cell lineages during injury[5] [6]. Moreover, the classical paradigm in which adult stem cells are restricted to differentiate into certain lineages has been recently challenged. However, the lack of reliable models, and inconsistency in the severity of intestinal injury, have yielded discrepant findings, so that the role of extra-intestinal cells to intestinal mucosal healing remains an open question.

To address this gap in our knowledge, we have employed a parabiosis system to establish a shared circulation between mice who were then subjected to either radiation-induced enteritis or chemically-induced colitis in order to investigate whether extra-intestinal cells can participate in the repair of injured intestine. We hypothesize that circulating cells originated from bone marrow participate in healing after both radiation-induced enteritis and chemically-induced colitis in parabiosis mice. Although the mechanisms providing protection against radiation-induced intestinal stem cell death and inflammatory bowel disease remain unknown, it is imperative to determine the contributing healing factors to moderate the excessive inflammatory process. While the ability to study circulating stem/progenitor cells and their potential role in intestinal healing remains limited by the lack of available animal models, a shared circulatory system in the parabiosis model allows us to investigate the previously unrecognized role of circulating cells and their effects in intestinal regeneration under physiological conditions.

While both radiation and TNBS are well-established injury models in the murine animal system, there are several concerns. Caveats of the irradiation-induced injury model include failure to properly protect the donor mouse from radiation. The lead shield that was used to cover the donor mouse may not provide enough protection during the irradiation session. When we monitored the amount of irradiation applied to the donor mouse by using a digital radiation monitor, it showed the donor mouse was exposed to 0.35 Gy (data not shown), which is negligible. This confirms that the donor partner is truly protected from the irradiation with the lead shield. To perform the experimental colitis injury model, TNBS was directly instilled into the colon to minimize the absorption of TNBS by the donor partner and to induce acute colitis only in an injured partner in a parabiosis system. Additionally, the functional, morphological, and histological analysis of the donor's intestine shows that confounding effects of TNBS absorption in the donor's gut is either minimal or undetectable.

Aim 2: To define the repair mechanisms of circulating stem cells in injured intestine.

AIM 2 INTRODUCTION

Stem cells belong to a specialized group of cells with distinct features—self-renewal and differentiating capacity. Depending on the aforementioned capacities, stem cells can be further grouped into gametes, embryonic cells, and adult stem cells. Adult stem cells are the most differentiated compared to other stem cells, with limited capacity to differentiate into only certain types of cells and to self-renew only for a sustained period. Recent evidence has shown that stem cells holds capacities to participate not only in the formation of tissues but also in their repair.

Bone marrow is the gelatinous center of the cortical bones, which contains two types of stem cells—hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). While both HSCs and MSCs are multipotent stem cells, they differentiate into different cell types. HSCs mainly support the components of the hematic system, producing blood cells including both myeloid and lymphoid lineage cells. In contrast, MSCs differentiate into smooth muscle cells, osteocytes, chondrocytes, and adipocytes. Utilizing multipotent capacities of bone marrow stem cells have been of great interest in regenerative medicine and cell-based therapy research due to relatively low ethical concerns compared to the use of embryonic stem cells.

Recent studies show evidence that stem/progenitor cells are found circulating in blood[35], indicating bone marrow could serve as a reservoir for circulating cells in peripheral blood. During physiological conditions, the number of progenitor cells in peripheral blood is extremely low—1 in 10^8 peripheral nucleated cells, resulting in challenges in isolating and characterizing an elusive stem cell population[12 36]. It is reported that circulating stem cells retain "primitive" or "stemness" properties, which decrease as humans age, explaining the inefficient healing process in the elderly population. The in-depth analysis of gene expression pattern of circulating progenitor cells shows the lower level of embryonic stemness genes activity in patients compared to healthy individuals, indicating that these cells may possess protective and repairing function[37].

Cell trafficking requires a directional migration of a cell towards a specific site, which is marked by chemoattractants. Circulating cells in blood stream recognize the chemoattractants released by immune cells at the injury site and home to the particular destination. Traditionally, it has been thought that progenitor cells found in peripheral blood are destined to migrate back to bone marrow and to contribute to the homeostasis of bone marrow stem cells. However, emerging evidence provides support for the idea that circulating progenitor cells translocate from marrow to various injured tissues in response to pro-inflammatory cytokines and chemokines[38]. We believe that release of cytokines, chemokines, and growth factors during pathological conditions increases the recruitment of bone marrow-derived cells, resulting the enhanced remodeling of the damaged tissue and restoring the intestinal lining.

We further hypothesize that through the development of a parabiosis system, which has stable and continuous source of bone marrow stem cells, we will test the ability of circulating stem cells to participate in intestinal healing after mucosal injury in two well-established injury models, namely radiation enteritis and chemical induced colitis.

The repair mechanisms of recruited bone marrow-derived cells include (1) direct replacement of damaged intestinal epithelial cells by the bone marrow-derived cells, (2) trans-differentiation of bone marrow-derived cells into intestinal stem cells, and/or (3) release of biomolecules from bone marrow-derived cells via the paracrine system.

Replacement of lost cells for restoration of damaged tissue structure is predominantly observed in organs with the limited proliferating capacity. The quiescent or non-dividing tissues including skeletal muscle and cardiac muscle are composed of terminally differentiated cells. Bone marrow-derived cells, specifically mesenchymal stem cells, contribute to tissue repair/remodeling by replacing damaged cells in certain organs, suggesting that they could be used to replace damaged or missing cells as clinical therapies for numerus diseases. It has been shown that injected mesenchymal stem cells differentiate into cardiac cells following myocardial infarction[39]. Furthermore, it is reported that cell fusion between bone marrow-derived cells and damaged tissue resident stem cells are observed even in the highly proliferating organ such as intestine.

On the other hand, peripheral blood-derived mesenchymal stem cells play a role as a new source of circulating osteogenic stem cells in the bone defect model[40]. Massive bone defect results from clinical trauma or metabolic disorder. It has been shown that

transplanted bone marrow cells home to the injury site and participate in the regeneration process, functioning as tissue specific stem cells. Injection of MSCs, the subpopulation of bone marrow stem cells, into osteogenesis imperfecta patients also promoted the bone growth.

In addition to cell restoration aspects in functional and structural recovery by recruited healing factors, trophic factors released from migrated cells promote regeneration at the injured site. Recently, it has been suggested that biomolecules secreted from recruited stem cells are can contribute as much to tissue repair as does the differentiation of stem cells into tissue specific cells. Multiple organs including intestines, skin, and bone marrow contain tissue-resident adult stem cells, which can be modulated by the surrounding microenvironment. Recruited stem cells of exogenous source can also regulate the niche for the resident stem cells by secreting chemokines, cytokines, and growth factors. In addition to potential cell proliferation effects, the paracrine mechanisms of recruited stem cells promote immunomodulatory pathways, thereby dampening harmful inflammation responses at the injury site. It has been shown that injection of bone marrow MSCs ameliorate the exaggerated inflammation progress in autoimmune disease.

In this study, we sought to investigate the mechanisms involved in mucosal healing and focused first on evaluating for the presence of circulating cells of bone marrow origin. Specifically, we suggest that a parabiotic partner will be protected against intestinal injury by translocation of healthy bone marrow-derived cells through the shared vasculature to the site of injury.

AIM 2 METHODS

Bone marrow cell transplant

In order to evaluate the effects of bone marrow cells on the healing response in the intestine, tdTomato^{+} total bone marrow cells, which were harvested from long bones of tdTomato donor mice, were injected into peripheral blood of the unpaired recipient via intraperitoneal injection. Presence of transplanted tdTomato^{+} cells in intestine was assessed by fluorescent microscopy. The unpaired recipient mice were 12 Gy gamma-irradiated 24 hours prior to transplant to cause intestinal damage and to eradicate the recipient's bone marrow stem cell population.

Harvesting and processing tissue specimens for intestinal Swiss roll

At the end of experimenting, small intestine or colon were harvested from the mice for Swiss roll sections. The entire colon or 10 cm of small intestine was cut longitudinally, rolled around a toothpick, and stabilized by 30G needle. Tissues were fixed overnight in 4% paraformaldehyde at 4°C, and washed three times in PBS. For paraffin sections, tissues were dehydrated and embedded in paraffin, and then sectioned at 5 um thickness. For cryosections, fixed tissues were incubated for 48 hours in 30% sucrose in PBS at 4°C and then embedded in optimal cutting temperature (OTC) medium. Sections were cut at 5-14 μm thickness.

Fluorescence Intensity Quantification

Fluorescence images were acquired with a Nikon Eclipse Ti Confocal microscope and x20 or x40 lens using NIS-Elements software at the same exposure level. Randomly selected sections were immunostained following strictly identical immunohistochemistry protocols. Measurements of fluorescence staining intensity was carried out with NIH ImageJ by converting color images to binary scale (fluorescence intensity from 0 to 255), and mean intensity in the region was calculated. Intensity was analyzed in 3-9 regions of the tissue in a single section per animal.

AIM 2 RESULTS

Bone marrow-derived cells home to the site of intestinal mucosal injury

We evaluated the migration of circulating bone marrow-derived cells into sites of intestinal inflammation. To do so, we first harvested bone marrow cells from tdTomato^{+} transgenic mice, and injected these peripherally into an unpaired recipient which had been 12 Gy gamma-irradiated 24 hours prior to transplant. Whole-body radiation was applied to both induce intestinal damage and to eradicate the recipient's bone marrow cell population. At day5 post-irradiation, tdTomato^{+} cells were detected in the lamina propria of the injured intestine, illustrating that bone marrow-derived cells could home to the injury site (Figure 2-1). We therefore next investigated whether bone marrow derived cells were seen in the other injury models, and if so, whether these cells contributed to the healing benefits of the parabiosis system. To do so, we first irradiated the WT parabionts which had been conjoined to a tdTomato mouse with 8 Gy gamma-irradiated to induce

the intestinal injury and ablation of the bone marrow cell population in the recipient. Three days after irradiation, numerous tdTomato^{+} cells from the paired mouse were observed in the lamina propria of the injured WT parabiont, indicating infiltration of bone marrow-derived cells of donor partner origin (Figure 2-1). It is noteworthy that in non-injured parabiosis controls, some numbers of tdTomato^{+} cells were also observed in the lamina propria despite the lack of intestinal inflammation, a finding which is consistent with a shared circulating cell population in the parabiosis system (Figure 2-1). We also observed the presence of bone marrow-derived cells into the colon in TNBS colitis injury model 3 days after TNBS instillation (Figure 2-1) showing that bone marrow-derived cells could be recruited and migrated to injury sites in both the models.

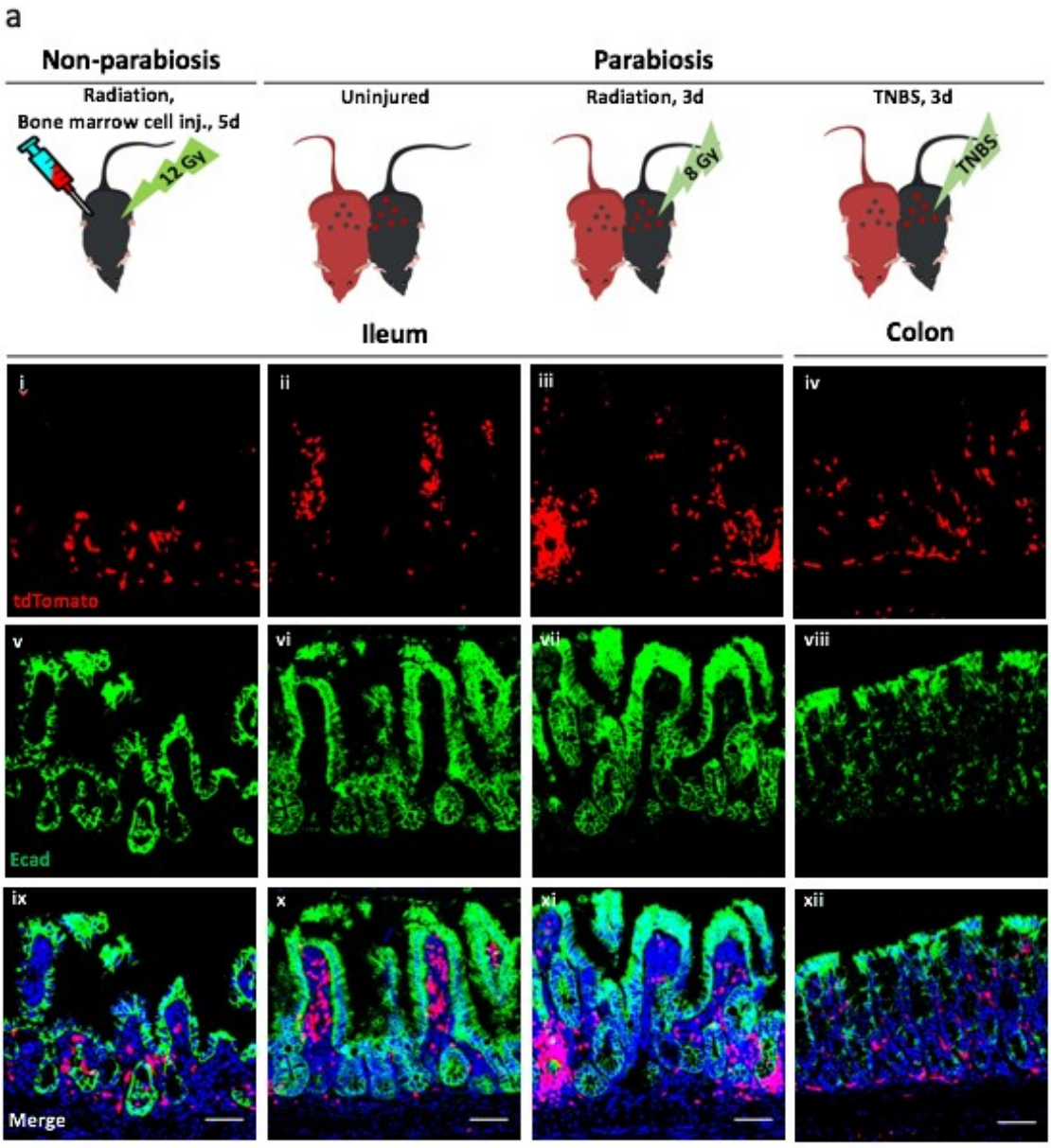


Figure 2-1. Circulating bone marrow-derived cells migrate to the injured intestine. (a) Representative images showing the double immunofluorescence of tdTomato and Ecad. Original magnification 20x; scale bars 100 µm.

It is noteworthy that the tdTomato cells did not co-localize with the epithelial marker e-cadherin in either mice that were injected with bone marrow derived cells or in parabiotic pairs subjected to models of radiation enteritis or TNBS colitis (Table 2-1). These findings suggest that trans-differentiation rarely occurs, suggesting a supportive role.

a

Group	% tdTomato^{+} Ecad^{+} cells
12 Gy, BMDC, Non-parabiosis	0.3
Uninjured, Parabiosis	2.2
8 Gy, Parabiosis	1.5
TNBS, Parabiosis	0.2

Table 2-1. Circulating bone marrow-derived cells rarely expresses an intestinal epithelial marker. (a) The percentage of tdTomato/Ecad-double positive cells out of total tdTomato+ cells shows the proportion of donor-derived intestinal epithelial cells in bone marrow-derived cell transplant recipients with 12 Gy radiation (n=2), uninjured parabionts (n=2 pairs), 8 Gy radiated parabiosis mice (n= 3 pairs), and TNBS-treated parabiosis mice (n=2 pairs).

Recruited bone marrow-derived cells preferentially congregate near the radiation damaged crypts during intestinal injury

We next explored the anatomic distribution of the bone marrow derived cells in the presence of intestinal injury. Unlike we initially expected, total number of recruited tdTomato$^+$ cells in the intestine of the irradiated parabiont was not significantly different compared to the parabiosis pairs with no radiation treatment (Figure 2-2). While there were no significant differences in the number of incorporated tdTomato$^+$ cells in the small intestine of an uninjured parabiosis pairs and 8 Gy irradiated parabiont, the distribution pattern of migrated tdTomato$^+$ cells in small intestine showed marked difference between a physiological and a pathological condition (Figure 2-2). In Swiss roll sections of an uninjured parabiont, tdTomato$^+$ cells were engrafted evenly in the lamina propria of villi and in submucosal region. However, tdTomato$^+$ cells in an parabiont injured by radiation were congregated in the stroma near the ionizing radiation damaged crypts, showing recruitment of bone marrow-derived cells to the injury site under pathological conditions.

To quantify the location of the bone marrow derived cells in the injured mucosa, we arbitrarily designated the upper part of villi as Zone 1 and the lower part of villi, in which proliferating intestinal stem cells reside, as Zone 2 (Figure 2-2). The quantification of tdTomato$^+$ cells depending on their location showed that significantly more tdTomato$^+$ cells are engrafted in Zone 2 in an irradiated parabiont (Figure 2-2). This suggests that homing signals from injured crypts may recruit circulating bone marrow-derived cells near the regenerating crypts, resulting in the changes of the distribution pattern of the bone marrow-derived cells during a disease condition.

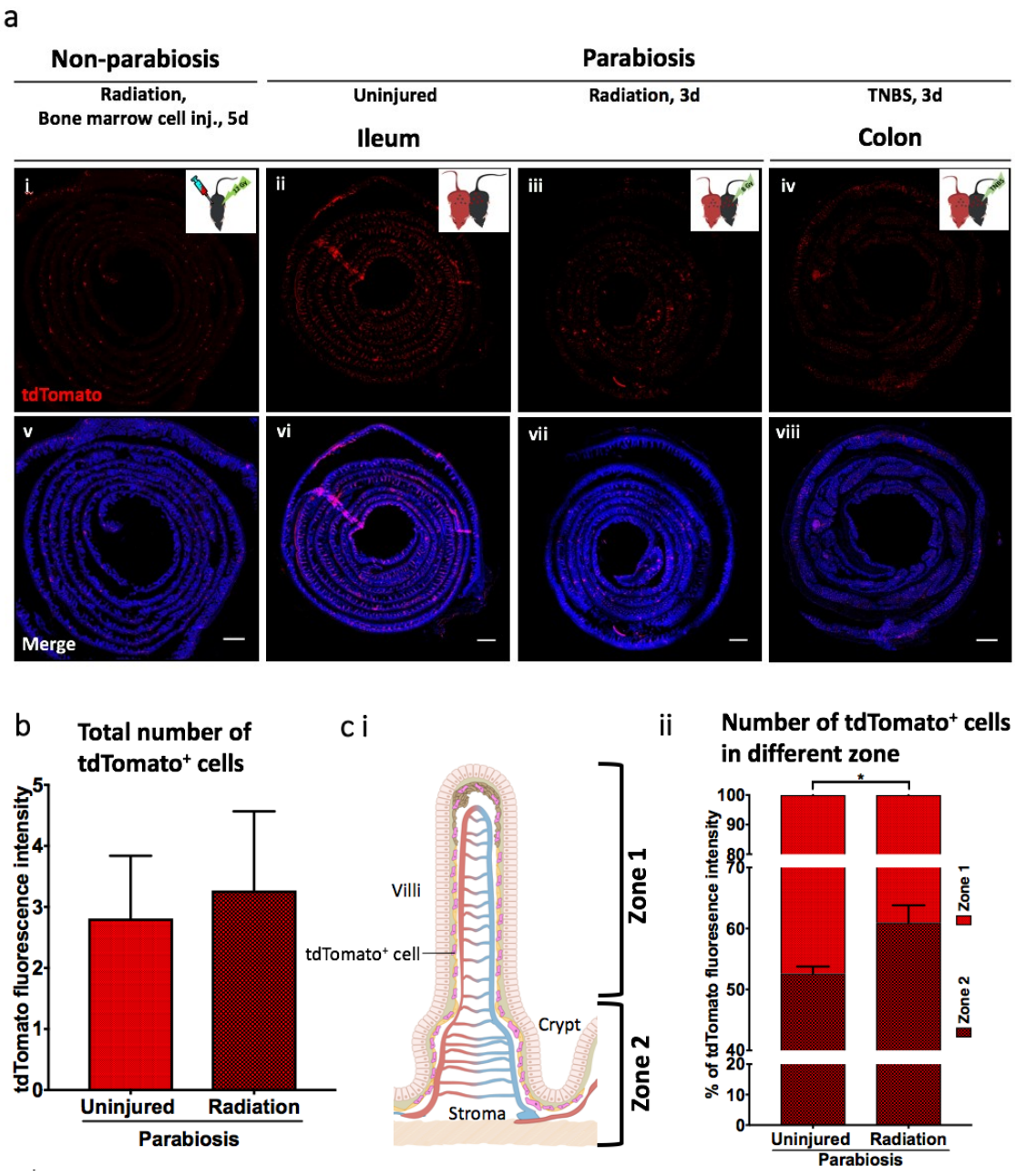


Figure 2-2. Circulating bone marrow-derived cells are congregate near the proliferating crypts. (a) Immunofluorescence analysis of tdTomato$^+$ cells in Swiss roll sections from bone marrow-derived cell transplant recipients with 12 Gy

radiation, uninjured parabionts, 8 Gy radiated parabiosis mice, and TNBS-treated parabiosis mice. (b) Quantification of fluorescence intensity of tdTomato$^+$ cells shows the number of recruited tdTomato$^+$ cells in small intestine of uninjured parabionts (n=3 pairs) and 8 Gy radiated parabiosis mice (n=3 pairs). Each value represents fluorescence intensity in 20x field of view of a single Swiss roll section. Results are presented as means ± SEM. (c) (i) The topographical designation of the upper part of villi as Zone 1 and the lower part, in which intestinal stem cells reside, as Zone 2. (ii) Quantification of tdTomato$^+$ cells depending on their location, Zone 1 *versus* Zone2, in uninjured parabionts (n=3 pairs) and 8 Gy radiated parabiosis mice (n=3 pairs). Results are presented as means ± SEM (*p < 0.05).

To delineate whether tdTomato$^+$ cells found in Zone 2 were simply repositioned from Zone 1 or migrated from bone marrow in response to crypt injury, the bone marrow cells were depleted in a parabiosis system by whole-body radiation of both parabionts. To show bone marrow plays a role as a reservoir for circulating cells in radiation enteritis, both parabionts in a parabiosis pair were radiated at 12 Gy. In a parabiosis pair, in which only one parabiont was radiated at 12 Gy, numerous tdTomato$^+$ cells were found in lamina propria of small intestine, which were migrated from a donor partner with intact bone marrow cell population. In contrast, in a parabiosis of both parabionts which were irradiated with 12 Gy, very few tdTomato+ cells were seen in the injured intestine (Figure 2-3). This result shows that tdTomato+ cells near crypts are recruited from bone

marrow in response to the homing signals under pathological conditions, rather than moving down from upper part of villi.

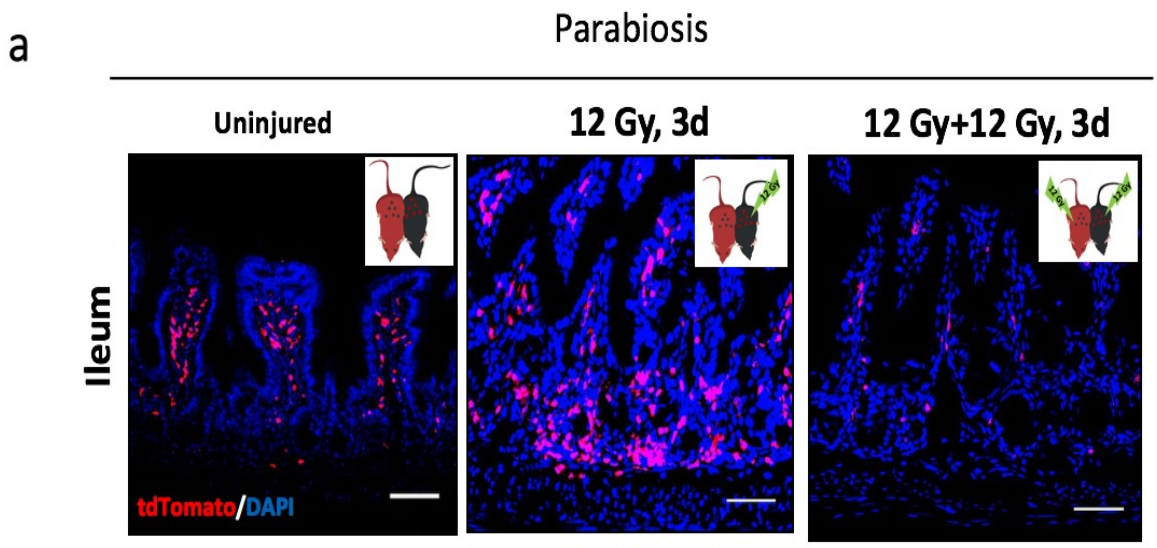


Figure 2-3. The depletion of bone marrow cells causes failure of recruitment to injured intestine. (a) Representative images of recruitment of tdTomato^{+} cells in small intestine of bone marrow-derived cell transplant recipients with 12 Gy radiation, uninjured parabionts, both parabionts which were irradiated with 12 Gy. Original magnification 20x; scale bars 100 µm.

AIM 2 DISCUSSION

In parabiosis system, a transgenic donor mouse expressing fluorescent reporter proteins allows us to trace all transplanted cells in a recipient mouse regardless of their origin or cell type. The above finding of abundant recruited tdTomato+ bone marrow-

derived cells in the injured intestine prompted us to test the cell fates of migrated bone marrow-derived cells in the injured intestine. We now report that circulating cells migrate to the sites of injury, are incorporated into the injured mucosa, and play a critical and previously unrecognized role in healing after mucosal injury in two complementary models. The recruited bone marrow-derived cells were observed in lamina propria, but not in the intestinal epithelial tissue, suggesting that the protective effects occurred via paracrine effects of the recruited bone marrow-derived cells, as opposed to trans-differentiation or direct engraftment. Our findings indicate that incorporated bone marrow-derived cells, which were found in the stroma near crypts, may interact with intestinal stem cells, playing a role as a paracrine mediator of intestinal inflammation. Additionally, lack of tdTomato^{+} cells in the injured intestine of bone marrow depleted parabiosis pairs strongly suggests that bone marrow-derived cells found in mesenchyme near crypts during intestinal injury were newly recruited from a donor parabiont following an acute injury.

Circulating stem cells respond to the distress signals from injured organs and stimulate the tissue repair and regeneration. While it is not fully understood how circulating progenitor cells migrate to the target tissue, several candidate mediators and chemotactic signaling pathways have been proposed. Stromal cell-derived factor-1 (SDF1)/CXC chemokine receptor-4 (CXCR4) axis has been intensely investigated in trafficking of mesenchymal stem cells. Binding of CXCR4, a G-protein-coupled receptor, with SDF1 activates signaling transduction pathways which are associated to chemotaxis and cell differenatiton[41]. It has been shown that ischemic tissues release SDF1 and bone

marrow-derived cells in peripheral blood are recruited to ischemic tissue in response to the chemoattractants, promoting revascularization and tissue regeneration[42]. While the chemokines induced by the intestinal injury may recruit circulating bone marrow-derived cells to the damaged crypts to create a supportive stem cell niche and induce mucosal healing, further investigation is needed to elucidate the homing/trafficking mechanisms of circulating progenitor cells in intestinal injury.

The current study extends the work of others who have examined the role of bone marrow-derived cells as an alternative source for replacement cells during injury, including studies demonstrating that bone marrow-derived cells participate in tissue regeneration by differentiating into organ-specific cells[43] [44] [45] [46]. Previous studies have mainly relied on bone marrow transplant or a transgenic mouse model with traceable genetic markers for specific cell populations to examine the role of bone marrow-derived cells in tissue repair/remodeling However, bone marrow transplantation does not assimilate the true physiological conditions of bone marrow, which makes it challenging to interpret the results accurately. Additionally, peripheral injection of bone marrow-derived cells does not allow us to assess if residential bone marrow stem cells have a capacity of egressing the bone marrow and directionally migrate toward injury sites.

Various studies have reported controversial, if not contradictory, results on bone marrow-derived cell engraftment in intestine following damages. Regarding the gastrointestinal tract, several studies have shown somewhat inconsistent findings, regarding both the potential of trans-differentiation of exogenous stem cells into intestinal tissue and the functional significance of these cells on tissue remodeling[14] [16]. Rizvi, A. Z

et al. have shown that transplanted bone marrow cells fuse with intestinal stem cell population, suggesting a critical role of cell fusion events in intestinal regeneration[14]. In contrast, de Jong's group has shown that the fusion event of intestinal epithelial cells with bone marrow derived cells is so rare that it is dispensable for intestinal repair process[16]. This contradiction could be explained by inherent limitation of bone marrow cell transplant protocol, which is commonly used in most of regenerative studies, and the unique anatomical feature of intestine. Peripheral injections of a finite number of bone marrow cells raises technical challenges in detecting limited numbers of injected cells in injured organs. Moreover, considering the small intestine is such a long organ (35 cm in adult mice), this rare cell population could easily go undetected during the scanning of a large portion of the small intestine in multiple sections. Conversely, drawing conclusions on the functional significance based on detecting a few events of cell fusion or trans-differentiation in such a lengthy organ should be cautioned, considering these observations may not be representative of entire tissue.

While it has been documented that bone marrow stem cells are capable of transdifferentiating into non-hematopoietic cells under disease conditions, in our study, the lack of events of tdTomato$^+$ cells co-expressing an epithelial cell marker in a lengthy Swiss roll section of small intestine and colon provides compelling evidence of supporting paracrine effects of the recruited circulating stem cells. The lack of differentiated intestinal cells which express tdTomato reporter protein and the topographical preference of tdTomato$^+$ cells near proliferating crypts during intestinal injury suggests that the protective effects occurred via paracrine effects of the recruited

circulating stem cells, as opposed to trans-differentiation. Considering gamma radiation preferentially targets rapidly proliferating intestinal stem cells in crypts, observation of dominant occupancy of migrated bone marrow-derived cells near injured crypts can be the governing repair mechanism in this specific injury model.

My thesis work utilizes a parabiosis model system, which warrants the continuous delivery of systemic bone marrow-derived cells to the injury sites under steady state, overcoming a major limitation of bone marrow transplantation, which delivers a finite number of bone marrow cells. As an unbiased approach, we used the Swiss roll preparation for histological sections, rolling up the intestine longitudinally, which allowed us to examine the whole section in a single slide. We show that lack of tdTomato^{+} villi or crypts even in bird's eye view of the Swiss roll section indicates the rarity of these events, as suggested by other investigators. However, it is noteworthy that the adverse effects on disease progress and clinical course after destruction of bone marrow cell populations clearly show that bone marrow-derived cells necessary for intestinal remodeling. Taken together, these findings indicate that the old paradigm of limited plasticity of stem cells may need to be revisited.

Aim 3: To characterize the migrated cell types in injured intestine promoting intestinal tissue repair.

AIM 3 INTRODUCTION

Although our knowledge on circulating stem cells is still incomplete, the characteristics of circulating stem cells are similar to those of bone marrow stem cells. Bone marrow contains two different types of stem cells —hematopoietic stem cells and mesenchymal stem cells. While hematopoietic stem cells differentiate into the lymphoid and myeloid lineage cells, mesenchymal stem cells are considered to give rise to various cell types, including osteoblasts, chondrocytes, adipocytes, and myocytes. The differentiation capacity of each stem cell population is reflected by the expression pattern of different cell surface markers. Sca-1, CD29, CD44, CD73, and/or CD105 are used for the positive identification for mouse mesenchymal stem cells, while mouse hematopoietic stem cells express CD45, CD117, and/or CD150. Negative markers for mouse mesenchymal stem cells are CD11b and/or CD45, while CD34, CD90, and/or Lin are used as negative markers to identify mouse hematopoietic stem cells.

It has been shown that the expression pattern of a set of surface markers is not largely different between circulating stem cells and bone marrow stem cells[12]. However, it has been shown that the expression pattern of CD73 on MSCs in peripheral blood is reduced, compared to MSCs in bone marrow, suggesting CD73 may be used as a differential cell marker which can delineate MSCs populations based on its origin.

To define characteristics of circulating stem cells during intestinal injury and regeneration, we tested a well-established cell surface marker of bone marrow stem cells, CD45. Since it was identified as a transmembrane protein tyrosine phosphatase, CD45 has been widely used as a pan-hematopoietic marker due to its specific, ubiquitous expression on most of hematopoietic lineage cells. While its molecular and cellular function is not clearly elucidated, it has been indicated in cell trafficking and homing mechanisms in leukocytes.

It is still unclear whether circulating stem/progenitor cells travel through the bloodstream or the lymphatic system. The blood is the major component of the circulatory system, circulating the oxygen and nutrients throughout the body. As a part of lymphatic system, lymph facilitates immunological responses and removal of metabolic waste products. The hematic and lymphatic have many parallel activities, which consist of interconnected, yet separate vessels. While blood vessels carry erythrocytes, leukocytes, and thrombocytes, the lymphatic vessels also provide an alternative route for leukocytes access to tissues, which is critical for modulating acute and chronic inflammation.

It is not exactly known how circulating stem cells respond to danger signals from the injured tissue, egress from bone marrow, and which routes are used to migrated to the target tissue. However, it is speculated that homing mechanisms of circulating stem cells may share similarities with the trafficking of hematopoietic stem cells to the marrow, or leukocytes to the inflammation sites. While endothelial cells express a range of different intergrins, gp38 (podoplanin) is selectively expressed in lymphatic endothelial cells[47 48].

While little is known about its biological function in lymphatic vessels, gp 38 may promote lymphatic tumor metastasis via increased tumor lymphangiogenesis. Hence, in this study, we tested the well-known lymphatic-specific marker, gp 38, to delineate the trafficking mechanisms of circulating stem cells in the injured intestine.

The major elements of intestinal lamina propria are mesenchymal cells, which have been gaining recognition as paracrine modulators in the inflammatory tissue. Stromal cells in the intestine reside in the submucosal compartment, interacting with epithelial cells and immune cells. While intestinal stromal cells have been viewed as a group of heterogeneous cells with a limited role in intestinal homeostasis, there has been a paradigm shift focusing on the novel role of stromal cells as a critical component in dynamic immunological networks. Stromal cells regulate the function of epithelial cells through the basement membrane. Release of biomolecules from stromal cells modulate the microenvironments around the intestinal stem cells, regulating the proliferation and differentiation of epithelium in intestines.

The intestinal stromal elements— myofibrobalsts, fibroblasts, pericytes, bone marrow-derived stromal stem cells, and the smooth muscle of the muscularis muscosae and the lymphatic lacteal— are identified by the expression of α smooth muscle actin (α-SMA)[49]. α-SMA is the microfilament component of intracellular cytoskeleton in intestinal stromal cells, and is the most specific marker for mesenchymal elements in intestine. There have been more reports on replenishment of intestinal mesenchymal elements by bone marrow stem cells, not only in liver[50] and lung[51], but also in injured intestine[52]. It has been reported that myofibroblasts of bone marrow origin were observed

in liver cirrhosis patients, suggesting contribution of circulating bone marrow cells to the repair process in liver. Given that α-SMA is considered the most reliable marker for the subepithelial myofibroblast elements[53], we tested whether migrated cells transdifferentiate into intestinal stromal elements and express α-SMA.

To investigate the identity and the homing mechanisms of migrated cells in injured intestine, we have conducted immunohistochemical staining of a well-known lymphatic vessel marker, hematopoietic stem cell marker, and stromal tissue marker in both bone marrow cell transplant recipients and 8 Gy radiated parabionts.

AIM 3 METHODS

Immunohistochemical staining

Immunohistochemistry was performed on 5-14 μm paraffin or cryosections as follows: cryosections were thawed at room temperature and rehydrated with PBS. Paraffin sections were first warmed to 63 °C in a vacuum incubator (Isotemp Vacuum Oven, Fisher Scientific) then washed immediately twice in xylene, gradually re-dehydrated in ethanol (100%, 95%, 70%, water), and then processed for antigen retrieval in citrate buffer (10mM pH6.0)/microwave (1000 watt, power level 10, 6 minutes). Samples were then washed with PBS, blocked with 1% BSA/5% donkey-serum (1 hour, room temperature), then incubated overnight at 4°C with anti-gp 38, -CD45, and -α-SMA primary antibodies (1:200 dilutions in 0.5% BSA, washed 3 times with PBS, incubated with appropriate fluorescent labeled secondary antibodies (1:1000 dilution in 0.5% BSA, Life Technologies Inc). All slides were incubated with the nuclear marker DAPI

(Biolegend), followed by multiple washes, and slides were then mounted using Gelvatol (Sigma-Aldrich) solution prior to imaging using a Nikon Eclipse Ti Confocal microscope under appropriate filter sets.

AIM 3 RESULTS

Non-hematopoietic lineage cells incorporate into intestinal stroma in bone marrow cell transplant recipients and injured parabionts via bloodstream

To show whether bone marrow-derived cells are recruited via blood circulation, rather than lymphatic system, we used gp38 (podoplanin), a selective marker for lymphatic endothelial cells,[54] in immunohistochemistry staining. We observed that tdTomato+ cells that were incorporated in lamina propria did not colocalize with gp38+ cells, indicating tdTomato+ cells may not circulate in the lymphatic system (Figure 3-1).

To test what hematopoietic lineage makers the migrated tdTomato+ cells express, a biological marker of hematopoietic stem cells (CD45) was used. CD45+tdTomato+ double positive cells were found lamina propria of non-injured parabiosis pairs, implicating the presence of CD45+ cells in lamina propria during physiological conditions (Figure 3-1). Interestingly, while tdTomato+ cells co-expressing CD45 were mainly found in the lamina propria of the upper villi (Zone1) in both bone marrow cell transplant recipients, which were 12 Gy irradiated, and 8 Gy irradiated parabionts, incorporated tdTomato+ cells which were localized near the crypts were negative for CD45 at the time point we assessed. Lack of co-localization of tdTomato+ cells in injured intestine for a mesenchymal stromal cell marker, α-smooth muscle actin (α-SMA),

indicates that migrated cells do not transdifferentiate into myofibroblasts at day3 post-radiation.

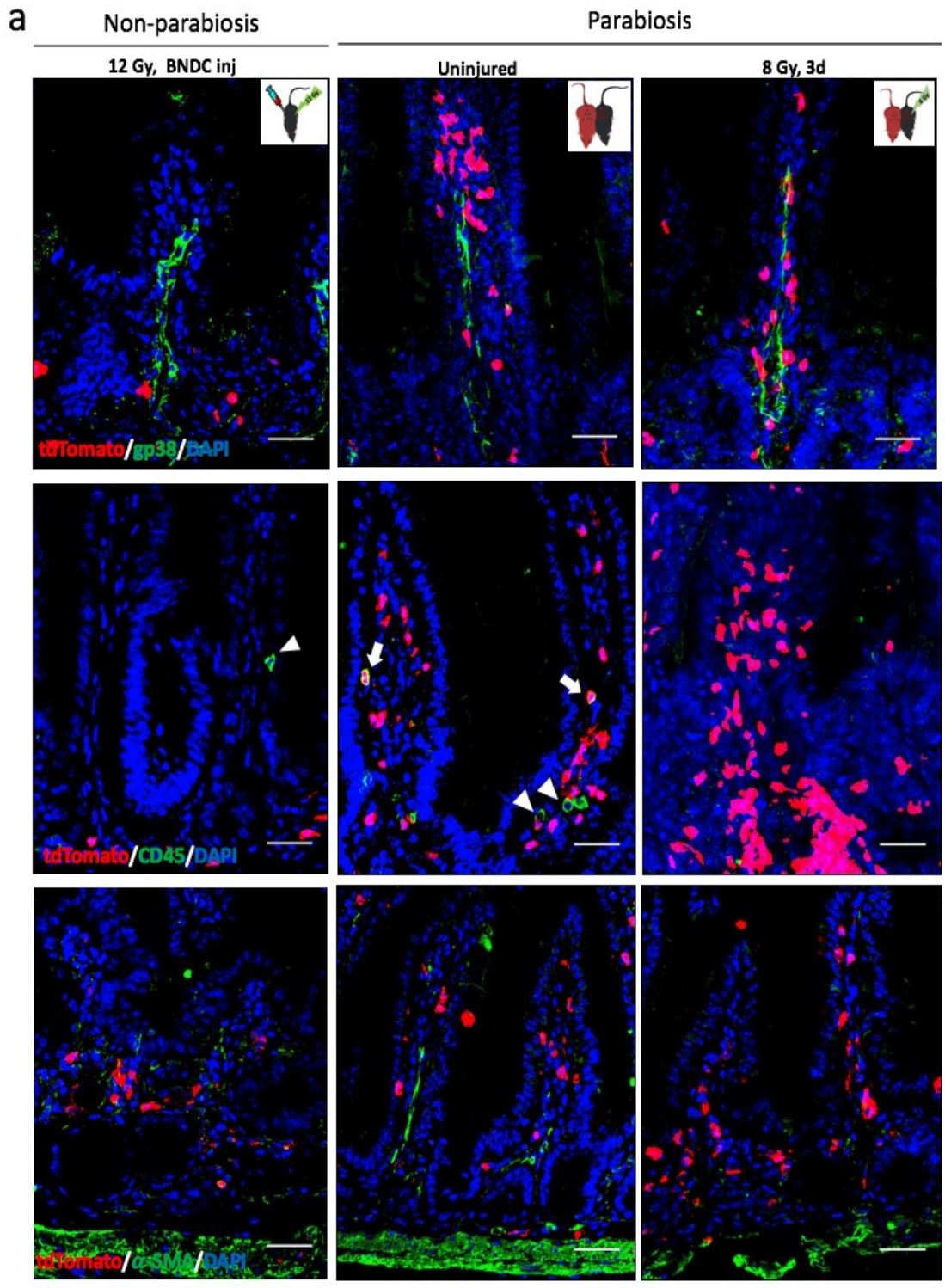

a
Non-parabiosis
Parabiosis
12 Gy, BNDC inj
Uninjured
8 Gy, 3d
tdTomato/gp38/DAPI
tdTomato/CD45/DAPI
tdTomato/αSMA/DAPI

Figure 3-1. CD45$^-$/α-SMA$^-$ cells from blood circulation incorporated into intestinal stroma in BMDC transplant and injured parabionts. (a) Representative images showing the double immunofluorescence analysis of tdTomato with gp38 (top), CD45 (middle), and α-SMA (bottom) staining from bone marrow-derived cell transplant recipients with 12 Gy radiation, uninjured parabionts, and 8 Gy radiated parabiosis mice. Arrows indicate double positive tdTomato$^+$/CD45$^+$ cells, and arrowheads indicate single positive tdTomato$^-$/CD45$^+$ cells in uninjured parabionts. Original magnification 40x; scale bars 50 μm.

AIM 3 DISCUSSION

Recent studies reported contradictory evidence on the recruitment of hematopoietic stem cells or non-hematopoietic stem cells to different organs in various disease models. The above results indicate non-hematopoietic derived cells are preferentially recruited to the ionizing radiation damaged crypts. These findings suggest localization of CD45$^-$ cells in intestinal mesenchyme proximal to crypts may play a major role in supporting intestinal stem cell function.

Despite the recent scientific advances in characterizing circulating stem cell, there are still significant challenges in identifying this cell population. While employing transgenic mice with a reporter marker for certain bone marrow stem cell populations, such as CD45 or Nestin, may provide an opportunity to evaluate the contribution of bone marrow-derived cells under steady state and during pathogenic conditions, these surface markers, often times, do not encompass the entire bone marrow cell population,

representing only a subset of population. Furthermore, upon exiting from the stem cell niche in bone marrow, the expression level of these genetic markers may change, resulting in failure of detection of migrated cells in the target organ. In spite of the reparative effect of transplanted bone marrow cells in injury sites, these cells may be short-lived, leaving a narrow window to trace these cells. To more reliably demonstrate the origin of healing factors, more extensive research would be needed, including identifying specific marker(s) for candidate stem cell population in specific tissue and developing transgenic mice expressing a reporter protein.

Taken together, our study shows that non-hematopoietic stem cells are favorably mobilized to the damaged gut under disease state, showing a functional significance of a subset of bone marrow stem cell population in radiation induced enteritis.

Aim 4: To investigate the functional consequences of pro-healing agents within the circulation and their depletion in intestinal regeneration.

AIM 4 INTRODUCTION

We next sought to investigate the functional consequences of eradication of the source for circulating stem cell in a donor parabiont of the parabiosis system by destroying bone marrow stem cells. Bone marrow is considered one of most sensitive organs to radiation[55]. Hematopoietic stem cells in marrow are highly susceptible to the ionizing radiation due to their high proliferation rate. However, mounting evidence has shown the different radiobiology of subpopulation of stem cells in bone marrow[55]. The bone marrow contains two distinct stem cell populations—hematopoietic stem cells and mesenchymal stem cells[56]. Radiosensitivity studies show that mesenchymal stem cells are more resistant to gamma radiation compared to hematopoietic stem cells[56]. It is suggested that a heightened activation of DNA double strand break repair pathways contribute to the relative radio-resistance in mesenchymal stem cells[57]. While low radiation doses of 2 Gy increase the level of senescence, no effects on apoptosis were observed[58]. In my thesis work, to eradicate the heterogeneous bone marrow stem cell population[59] with minimal systemic effects[60], the donor parabiont was exposed to the whole body 4 Gy radiation. We hypothesize that the rescue effects such as restored crypts and preserved epithelium will be averted even in a parabiosis system when the donor partner's bone marrow stem

cells are depleted by 4 Gy of radiation. To test our hypothesis, we investigated the functional relevance of bone marrow in intestinal repair and regeneration in the acute colitis injury model.

It has been reported excessive reactive oxygen species and reactive nitrogen species are observed in IBD patients[61 62]. Superoxide (O2(·-)) and nitric oxide (NO) are produced by activated inflammatory cells. While the release of toxic defense molecules by innate immune cells inhibits pathogen replication, these tissue-damaging species recruit more effector cells to the inflammation sites, further exacerbating the mucosal inflammation[63]. 3-Nitrotyrosine (3-NT) is a product of tyrosine nitration mediated by reactive nitrogen species such as NO[64]. 3-NT is identified as a biomarker of cell damage, inflammation, and NO production. The elevated level of 3-NT under pathological conditions, including IBD, indicates that it can be utilized as a specific injury marker of oxidative damage[65]. The present study investigated the involvement of 3-NT, a biomarker of NO in TNBS-induced colitis.

Whole transcriptome sequencing such as RNA-Seq is a powerful tool for analyzing the transcriptome of a biological sample and identifying biological networks which regulate the pathological process during disease conditions[66 67]. With a careful assessment of transcriptional changes of biological samples, we can examine which genes are differentially expressed and which signaling pathways regulate the disease progression. While whole transcriptome sequencing provides comprehensive understating in complex biological networks and interactions, it creates very large data sets and requires high-technology equipment with big storage memory to appropriately process

and analyze data[68]. On the other hand, RNA-Seq using targeted RNA panels quantifies gene expression profiling of biologically- and disease-relevant pathways, with minimal statistical and bioinformatic processing. This approach is capable of identifying significant differences in gene expression within the pre-selected transcriptome and allows us to focus on most relevant signaling network analyses. To elucidate signaling pathways involved in cellular and molecular response to gamma radiation, we examined over four hundred representative genetic components of multiple canonical pathways associated with the protection against radiation enteritis in a parabiosis system. In this study, we investigated the gene pathway relationships in 8 Gy radiated unpaired mice and parabiosis mice to identify interesting gene network regulation.

AIM 4 METHODS

Flow cytometry

Bone marrow cells were flushed with PBS from femur and tibia of WT mice. After removing red blood cells by incubation in lysis buffer, cells were passed through a 40-μm cell strainer for single cell suspension. After centrifugation at 400 × g at 4°C for 5 min, the pellet was then resuspended in 1% BSA in PBS and centrifuged at 400 × g at 4°C for 5 min and the supernatant was discarded. For cell surface marker staining, single-cell suspensions were then incubated with anti-CD16/CD32 (BD Biosciences) to block Fc receptor binding (20 min, 4°C). Cells were pelleted by centrifugation and resuspended in primary fluorochrome-conjugated antibody: CD45(I3/2.3, Biolegend) in FACS buffer.

BD Accuri C6 flow cytometer was used to collect flow cytometry data. Data analysis was performed using FlowJo software.

RNA sequencing

Total RNA from the small intestine was purified and cDNA library was generated by Mouse Signal Transduction Pathway Finder (QIAseq Targeted RNA Panels, Qiagen). Sequencing was performed on the Illumina MiSeq, dedicating minimum 1.4 million reads per sample. All RNA-Sequencing files (.fastq) passed quality control metrics and were analyzed at QIAGEN's GeneGlobe portal. The ten reference gene assays (MAP3K2, FBRSL1, KIAA0586, THAP3, C16orf13, RFX1, PPIE, ZNF446, PPIL2, ZBTB22) were used to normalize data.

AIM 4 RESULTS

Eradication of a putative source for circulating bone marrow-derived cells leads to severe aggravated intestinal inflammation in injured parabiosis

A low dosage of 4 Gy whole-body irradiation was administered to the tdTomato parabiont 4 days prior to TNBS administration to the WT parabiont. (Figure 4-1).

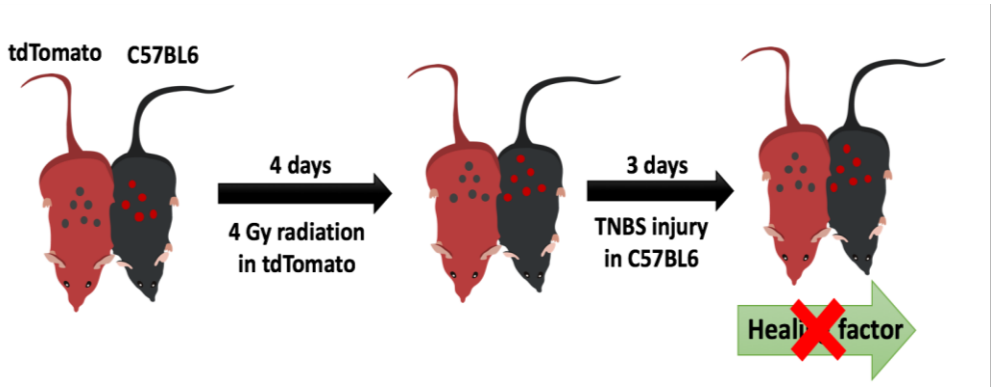


Figure 4-1. Graphical representation of bone marrow cell eradiation in the murine parabiosis model prior to TNBS treatment.

Ablation of the bone marrow by 4 Gy dosage was confirmed by flow cytometry analysis in 4 Gy radiated unpaired mice (Figure 4-2). At 4 day post-radiation, the fraction of total bone marrow cells were reduced in half (from 62% to 34%). The number of $CD45^-$ cells in marrow was also reduced in half (from 2200 cells to 1300 cells) after whole body 4 Gy radiation (Figure 4-2).

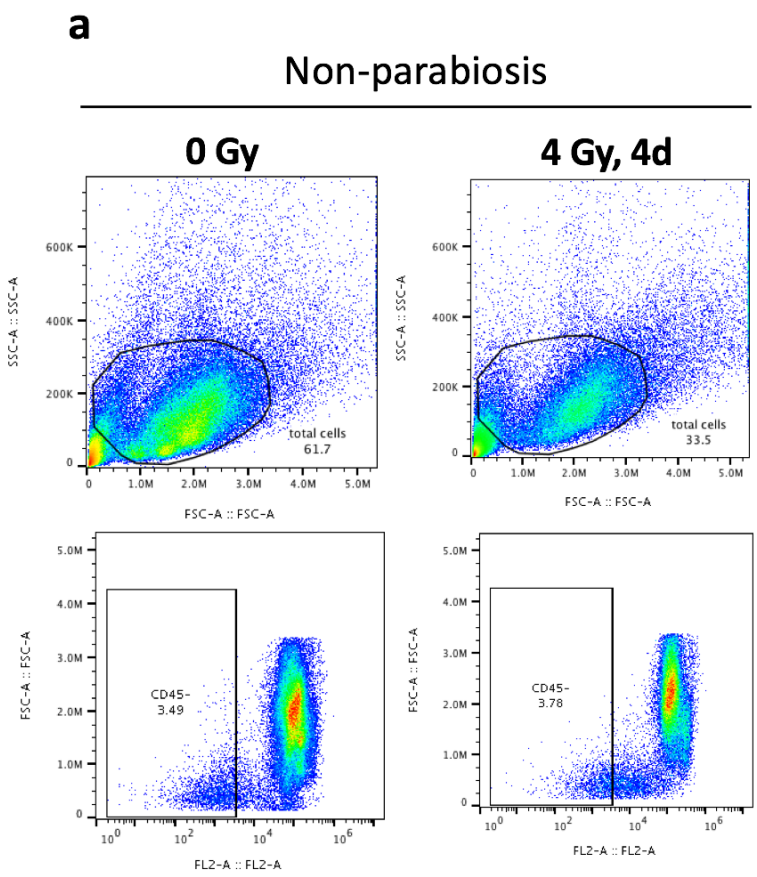
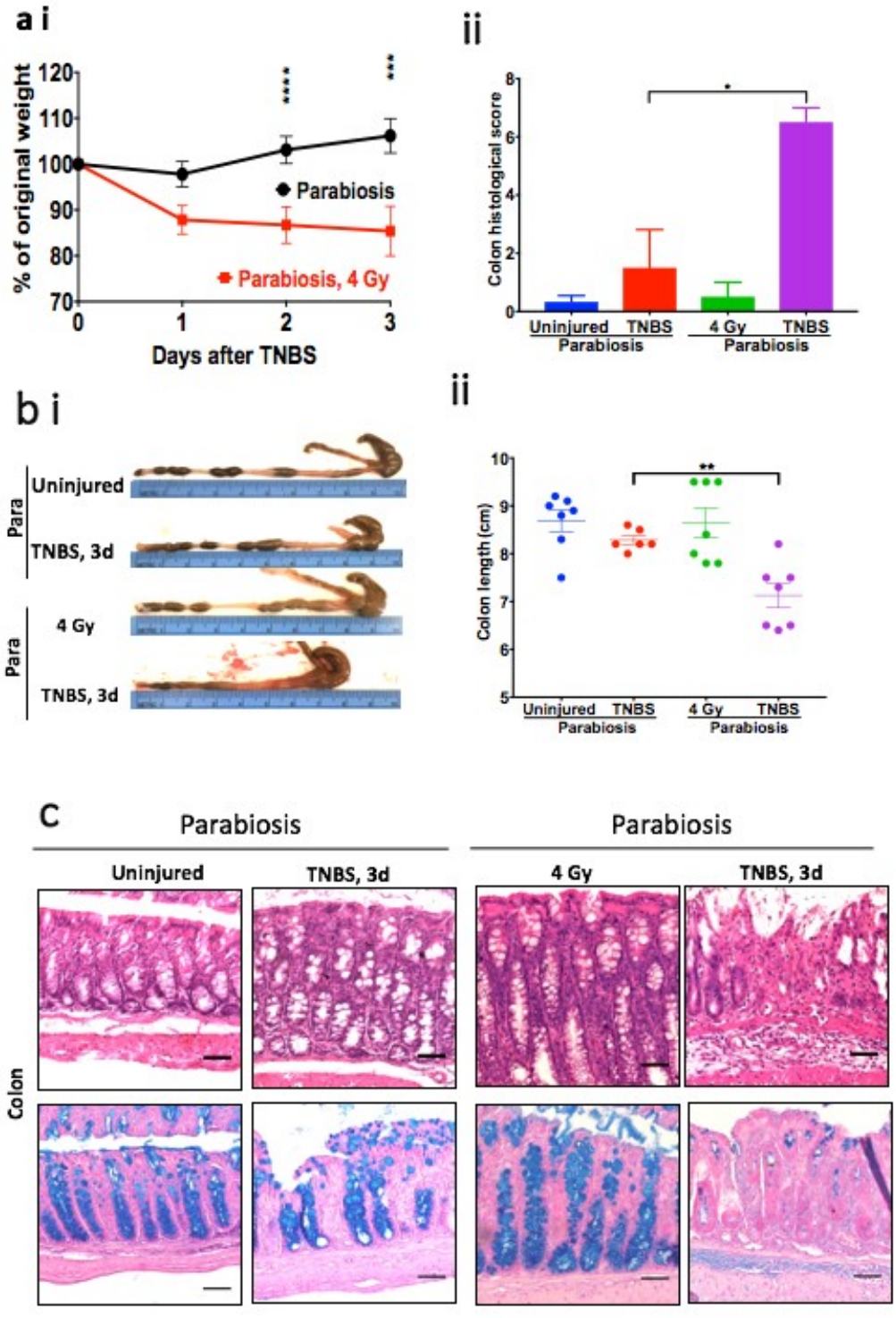


Figure 4-2. 4 Gy whole-body radiation depletes CD45- bone marrow cell population in marrow. (a) Flow cytometry analysis shows the percentage of total cells in bone marrow (top); CD45- cells in bone marrow (bottom).

Most strikingly, a TNBS-treated parabiont who was conjoined to the 4 Gy irradiated mouse displayed worse clinical course, manifesting significantly more body weight loss at day 2 and 3 compared to TNBS-treated parabiosis mice (Figure 4-3). The colon of TNBS-received parabionts conjoined to a donor partner with compromised bone

marrow cells was significantly short and thickened due to extensive inflammation compared to TNBS-received parabionts (Figure 4-3). Histological examination based on the aforementioned scoring system also illustrates the low dosage of radiation on the donor parabiont prevented the previously observed rescue effects in a parabiosis system (Figure 4-3).


a i
% of original weight
120
110
100
90
80
70
0
1
2
3
Days after TNBS
****
***
Parabiosis
Parabiosis, 4 Gy
ii
Colon histological score
8
6
4
2
0
*
Uninjured
TNBS
4 Gy
TNBS
Parabiosis
Parabiosis
b i
Para
Uninjured
TNBS, 3d
Para
4 Gy
TNBS, 3d
ii
Colon length (cm)
10
9
8
7
6
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4 Gy
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Parabiosis
Uninjured
TNBS, 3d
4 Gy
TNBS, 3d
Colon

Figure 4-3. Eradication of a putative source for circulating BMDC leads to worse clinical course in TNBS-induced colitis. (a)(i) Body weight change in TNBS-received parabiont mice without radiation (n=14 pairs) and with prior 4 Gy radiation (n=5 pairs). Data are presented as means ± SEM. (ii) Histological damage level of TNBS treated parabionts with or without 4 Gy radiation was scored in a blind manner. Parabiont mouse without radiation (n=3 pairs) and parabiont mouse with 4 Gy radiation (n=3 pairs) from TNBS injury model. Data are presented as means ± SEM. (b)(i) Macroscopic images of colons from TNBS-received parabiont mice without radiation and with prior 4 Gy radiation. (ii) Colon length of TNBS-received parabiont mice without radiation (n=6-8 pairs) and with prior 4 Gy radiation (n=7 pairs). Data are presented as means ± SEM. (c) Representative H&E-stained sections from TNBS-received parabiont mice without radiation and with prior 4 Gy radiation (top); Representative Alcian blue-stained sections from TNBS-received parabiont mice without radiation and with prior 4 Gy radiation (bottom). Original magnification 20x; scale bars 100 μm.

RT-qPCR (TNF-α, IL-1β, Lipocalin2, IL-6, CCL2, CXCL2, Lipocalin2) demonstrates that although two mice established the shared circulation, loss of bone marrow cells resulted in increased level of inflammation and colonic mucosal damage in a parabiosis system (Figure 4-4). The levels of cellular apoptosis and proliferation also indicate the rescue effects are reversed in injured parabionts who are conjoined with the bone marrow cell depleted partner (Figure 4-4).

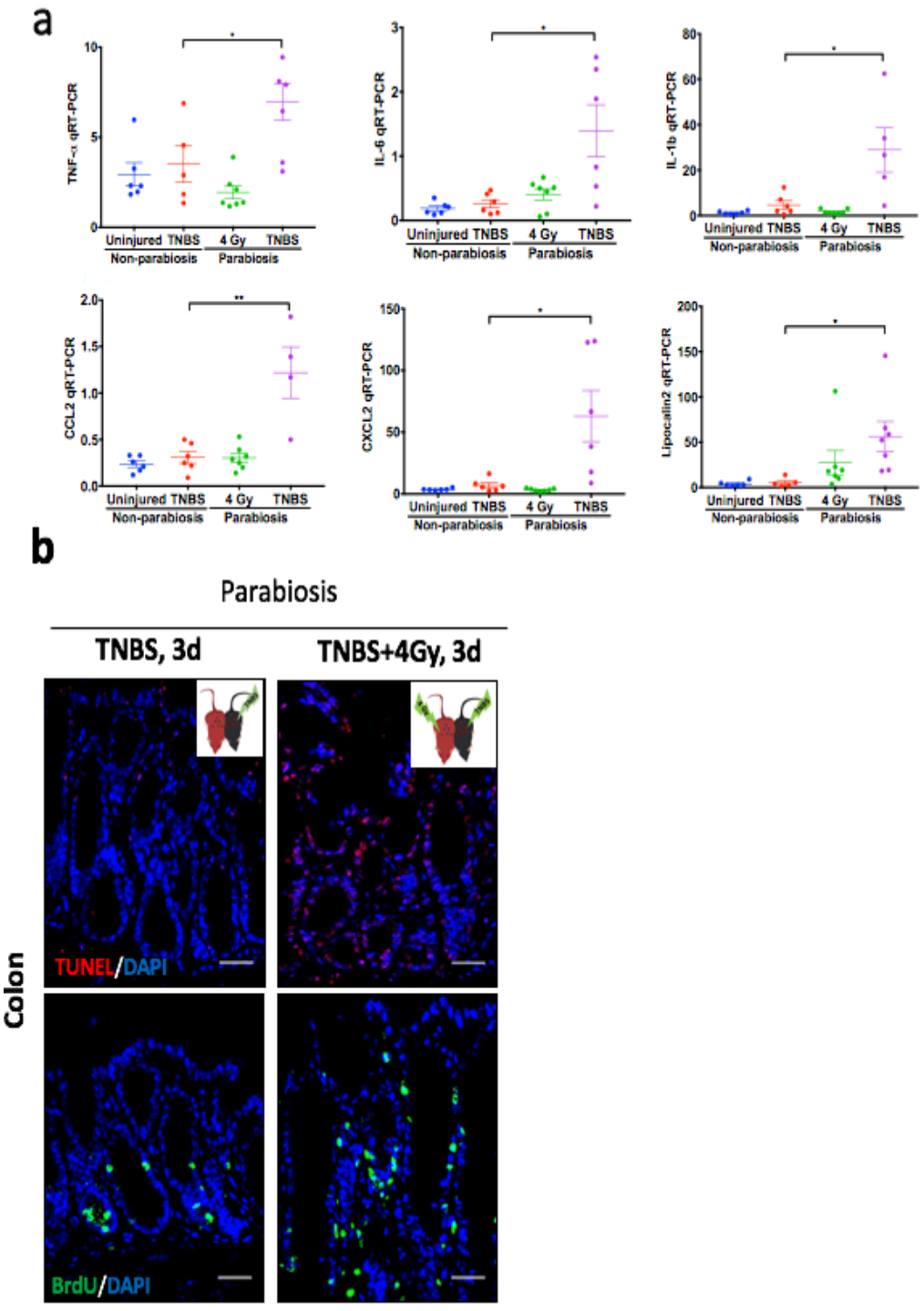

a
TNF-α qRT-PCR
IL-6 qRT-PCR
IL-1b qRT-PCR
CCL2 qRT-PCR
CXCL2 qRT-PCR
Lipocalin2 qRT-PCR
Uninjured
TNBS
4 Gy
TNBS
Non-parabiosis
Parabiosis
b
Parabiosis
TNBS, 3d
TNBS+4Gy, 3d
Colon
TUNEL/DAPI
BrdU/DAPI

Figure 4-4. Eradication of a putative source for circulating BMDC leads to severe aggravated intestinal inflammation in TNBS-induced colitis. (a) RT-qPCR of in TNBS-received parabiont mice without radiation (n=5-6 pairs as indicated) and with prior 4 Gy radiation (n=7 pairs). Data are shown as Mean ± SEM. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) (b) Representative immunohistochemistry images of TUNEL (top) and BrdU (bottom) from TNBS-received parabiont mice without radiation. Original magnification 40x; scale bars 50 μm. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

Immunochemistry reveals the elevated level of 3-NT in TNBS-treated non-parabiosis mice and TNBS-received parabionts conjoined with bone marrow cell depleted partners (Figure 4-5). In contrast, in TNBS-received parabionts, 3-NT expression in colon was considerably lower. These findings suggest that highly activated reactive nitrogen species are involved in intestinal inflammation and bone marrow-derived cell may modulate the nitrative stress in acute colitis.

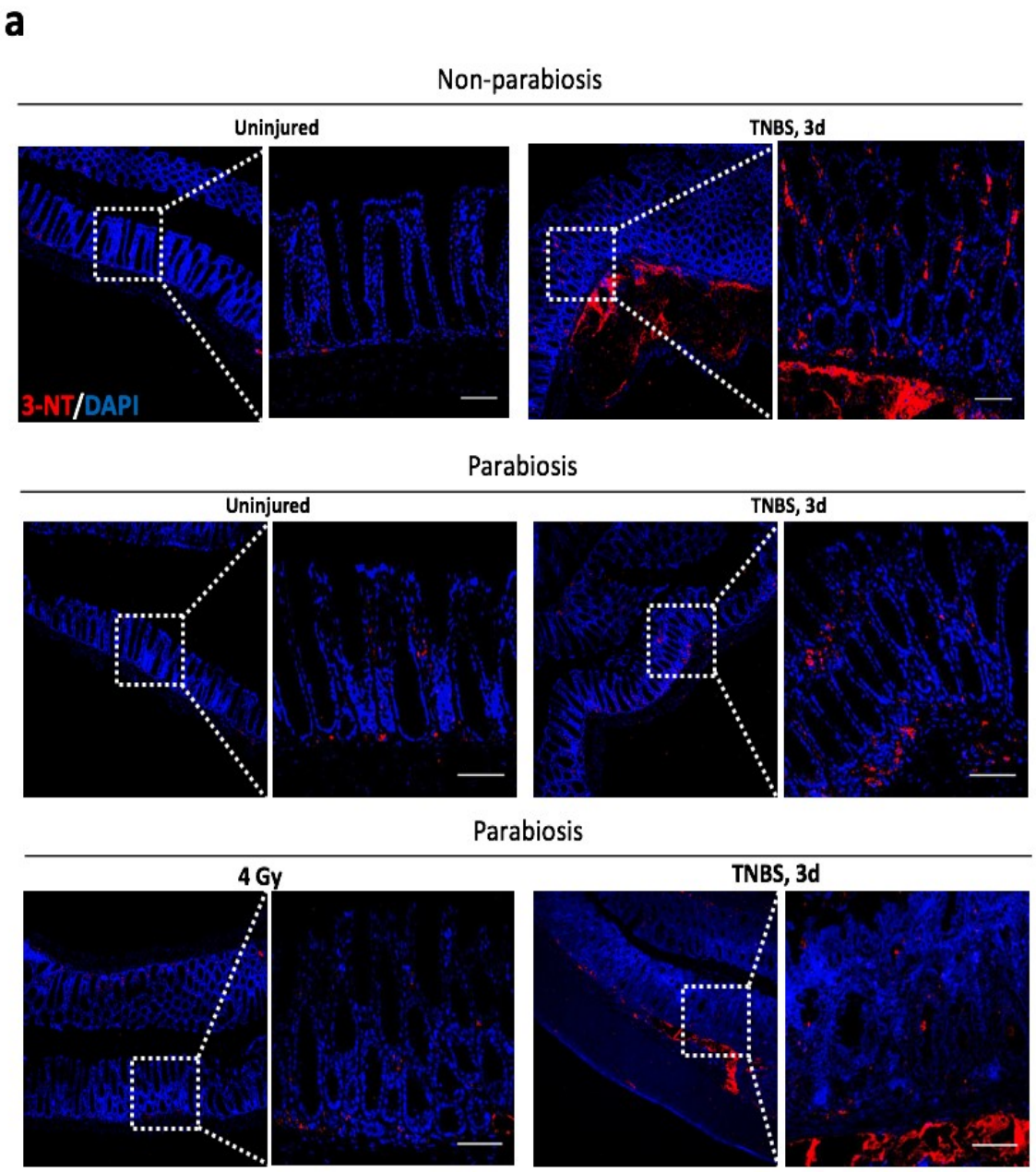
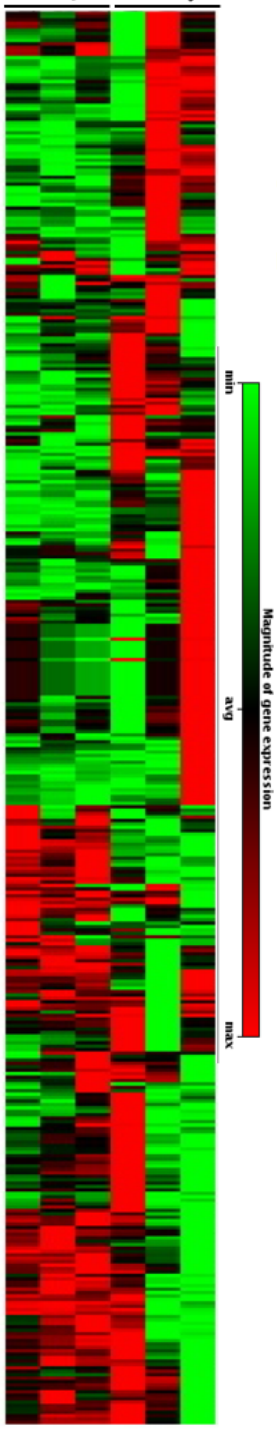


Figure 4-5. iNOS damage is ameliorated in TNBS-treated parabiosis system with an intact bone marrow cells population. (a) Representative immunohistochemical staining of 3-NT from colon of TNBS injury model. The right panel of each group is

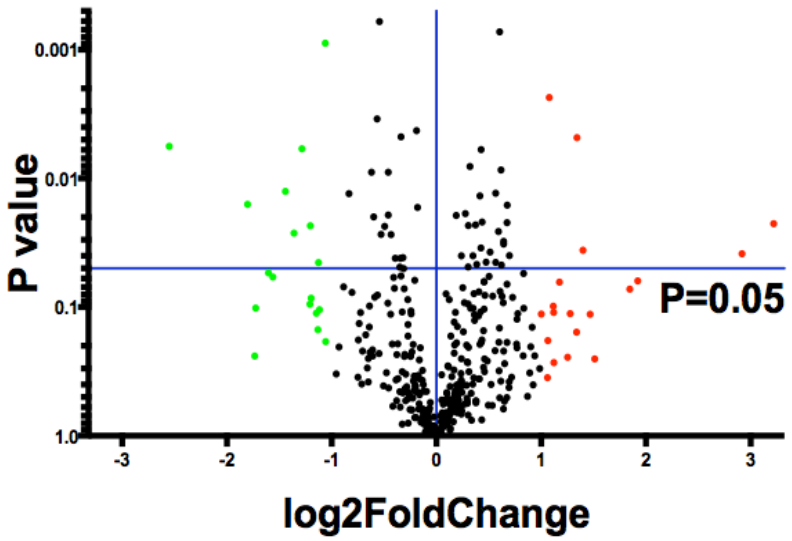
magnified views of the boxed area in the left panel. Original magnification 10x for the left panels and 20x for right panels; scale bars 50 μm.

Sequencing the expression patterns of over 400 genes in canonical signaling pathways, we identified 19 upregulated and 18 downregulated genes in 8 Gy radiated parabiosis intestine, compared to the 8 Gy radiated unpaired mice (Figure 4-6). These findings were also verified by RT-qPCR. Analysis of signaling transduction pathways by RNA-Seq in irradiated parabiosis mice suggest possible molecular pathways that contribute to the protection against radiation enteritis.

a



b



c

Gene Symbol	Fold Regulation	p-value	Gene description
Angptl4	2.1	0.353	Angiopoietin-like
Apoc3	2.1	0.182	Apolipoprotein C-III
Ckm	2.3	0.064	Creatine kinase, muscle
Cyp1a1	9.3	0.022	Cytochrome P450, family 1, subfamily a, polypeptide 1
Cyp4v3	2.6	0.036	Cytochrome P450, family 4, subfamily v, polypeptide 3
Fasn	2.5	0.157	Fatty acid synthase
Fcer2a	2.0	0.113	Fc receptor, IgE, low affinity II, alpha polypeptide
Fkbp5	2.5	0.005	FK506 binding protein 5
G6pc	3.8	0.063	Glucose-6-phosphatase, catalytic
Gck	2.2	0.270	Glucokinase
Heyl	3.6	0.072	Hairy/enhancer-of-split related with YRPW motif-like
Irs2	2.1	0.002	Insulin receptor substrate 2
Lef1	2.4	0.112	Lymphoid enhancer binding factor 1
Mme	2.2	0.110	Membrane metallo endopeptidase
Ppargc1a	2.8	0.114	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Rnf144b	2.2	0.098	Ring finger protein 144B
Sgk1	2.8	0.252	Serum/glucocorticoid regulated kinase 1
Slc6a12	2.4	0.245	Solute carrier family 6 (neurotransmitter transporter), member 12
Zbtb16	7.6	0.038	Zinc finger and BTB domain containing 16
Ada	-2.3	0.023	Adenosine deaminase
Ccl2	-2.6	0.027	Chemokine (C-C motif) ligand 2
Ccl5	-3.0	0.058	Chemokine (C-C motif) ligand 5
Cxcl2	-3.0	0.054	Chemokine (C-X-C motif) ligand 2
Cxcl3	-3.3	0.102	Chemokine (C-X-C motif) ligand 3
Fosl1	-2.2	0.045	Fos-like antigen 1
H19	0.0	0.016	RNA Gene, affiliated with the non-coding RNA class
Il1b	-2.1	0.185	Interleukin 1 beta
Lep	-3.3	0.240	Leptin
Olr1	-2.2	0.150	Oxidized low density lipoprotein (lectin-like) receptor 1
Pgc	-2.2	0.105	Progastricsin (pepsinogen C)
Pgr	-2.3	0.095	Progesterone receptor
Rbp1	-2.4	0.006	Retinol binding protein 1, cellular
Serpina3n	-2.7	0.013	Serine (or cysteine) peptidase inhibitor, clade A, member 3N
Slc7a11	-2.2	0.112	Solute carrier family 7 (cationic amino acid transporter), member 11
Stra6	-2.1	0.001	Stimulated by retinoic acid gene 6
Tnf	-2.3	0.085	Tumor necrosis factor
Ubd	-5.9	0.006	Ubiquitin D

Figure 4-6. Gene expression profiling of signal transduction pathways in irradiated small intestine. (a) RNA-Seq heat map summarizing gene expression patterns of day-3 8 Gy irradiated non-parabiosis mice and parabionts. Up-regulation is indicated by the red color, and downregulation is indicated by green color. The genes are clustered according to their expression patterns. (b) A volcano plot of differentially expressed genes. Positive log2 (fold change) values represent upregulation in radiated parabionts compared to radiated unpaired mice, and negative values represent downregulation. (c) The list of differentially expressed genes. The fold change of each gene is calculated in respect to the expression profile of a pool (n=3) of radiated parabionts versus radiated non-parabiosis mice (n=3).

AIM 4 DISCUSSION

To demonstrate the functional consequences of the loss of a putative source for circulating healing factors in acute colitis, we used gamma irradiation to destroy bone marrow stem cells, which are highly sensitive to radiation. When the donor partner's bone marrow stem cells are depleted by a low dosage of radiation, we don't observe the rescue effects even in a parabiosis system. These mice, which lack bone marrow stem cells, manifested worse intestinal inflammation. In the absence of bone marrow stem cells in the donor partner, TNBS-treated mice, which were conjoined to the irradiated donor partner, exhibited more severe colitis and intestinal damage. This indicates that bone marrow-derived cells exited from the bone marrow play critical roles in intestinal regeneration and repair in TNBS-induced colitis, suggesting the possibility that

upregulating the mobilization of patient's own bone marrow stem cell population could be a novel therapeutic approaches for these diseases. While bone marrow cell population in TNBS-treated parabiont mice is still intact, these bone marrow cells fail to provide the protection in TNBS colitis injury model. This can be explained that bone marrow cells may regulate the homeostatic repair and damage, but larger quantity of bone marrow cells are required to mitigate the pathological conditions, which were provided from a donor parabiont.

These results illustrate that absence of bone marrow cells leads to severe aggravated intestinal inflammation even in a parabiosis system. This apparent loss of protection in intestinal inflammation in a parabiosis system suggests that having an intact bone marrow cell population is required in enhanced recovery in acute colitis. Furthermore, bone marrow-derived cells may ameliorate the pathologies of the acute colitis by reducing nitroxidative stress. Together, these findings help to further establish the clinical relevance and translational significance of signaling pathway transcriptome sequencing in the parabiosis model system.

Although both bone marrow-derived cells transplant experiment and parabiosis system data suggest that the circulating healing factors are originated from marrow, bone marrow may not be the only source of the humoral healing factors[69]. It has been reported by several researchers that multipotent mesenchymal stem cells (MSC), which can contribute to regeneration in some organs, can be isolated from not only bone marrow but also adipose tissue[70 71 72]. To more reliably demonstrate the origin of circulating stem cells, more extensive research would be needed, including identifying specific marker(s)

for candidate stem cell populations in specific tissues and developing transgenic mice system to facilitate lineage tracing. While confirmation of the lack of rescue effects after bone marrow cell destruction in the TNBS system demonstrates the direct involvement of exogenous cell-mediated protection from intestinal inflammation, we cannot rule out the contribution of non-cellular healing factors from peripheral circulation. It has been shown that circulating anti-inflammatory cytokine IL-10 in serum has immunomodulating effects in IBD[73]. The beneficial effects of bone marrow-derived cells and the mechanisms of their anti-inflammatory activity after intestinal damage requires further elucidation.

Recent studies have shown that the involvement of pro-inflammatory cytokines and chemokines in intestinal inflammation[74]. Progressive inflammation results in increased cell death, which can lead to irreversible tissue damage, contributing to the severity of the disease. Our findings extend the findings of others who have used the parabiosis system to elucidate the mechanisms underlying healing from mucosal injury. In particular, Watanabe M.[75 76 77] and Hibi T. [78 79 76 80 77] group demonstrates the complexity of gut homeostasis under steady state and pathologic responses under chronic colitis. A parabiosis system was used to assess whether mucosal or systemic IL-17 is required to develop colitis. While IL-17 was generally considered as colitogenic cytokine, surprisingly, intestinal IL-17 was not essential to develop chronic colitis. Furthermore, the conventional dogma on pathological role of Th17 cells in chronic colitis requires further investigation. During pathological conditions, massive infiltration of Th17 cells in chronic colitis patients' colon triggers the inflammatory process. However, naturally occurring Th17 cells suppress the development of colitis, showing, functional plasticity

of Th17 in homeostasis state. These findings suggest complex mucosal regeneration mechanisms are involved in intestine under steady state and injury.

In this study, we conclude that circulating cells of bone marrow origin can facilitate intestinal healing using a parabiotic system, suggesting novel cellular approaches to the treatment of intestinal injury. Our approach of utilizing comprehensive injury models, encompassing not only small intestine and large intestine, but also intestinal stem cells and differentiated epithelial cells, helps us to further delineate how circulating factors home to the gut and aid the healing process. These experiments increase our understanding of the healing process of damaged gut and shed light on the utilization of circulating bone marrow-born cells as a possible therapy for intestinal diseases.

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14. Rizvi, A. Z. *et al.* Bone marrow-derived cells fuse with normal and transformed intestinal stem cells. *Proc. Natl. Acad. Sci.* **103**, 6321–6325 (2006).
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29. Yamasaki, S. *et al.* Circulating nucleated peripheral blood cells contribute to early-phase meniscal healing. *J. Tissue Eng. Regen. Med.* **11**, 609–617 (2017).
30. Li, C. *et al.* RhoA determines lineage fate of mesenchymal stem cells by modulating CTGF–VEGF complex in extracellular matrix. *Nat. Commun.* **7**, 11455 (2016).
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Curriculum Vitae

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EDUCATION

Johns Hopkins School of Medicine, Baltimore, Maryland 2015-2018
Ph.D./ Human Genetics

University of North Carolina, Pembroke, North Carolina 2011-2012
B.S./ Biology

Ewha Womans University, Seoul, South Korea 1995-1999
B.S.N./ Nursing Science

LICENSURES/CERTIFICATIONS

Registered Nurse, Multistate, USA
Advanced Cardiac Life Support (ACLS) certified by American Heart Association
Basic Life Support (BLS) certified by American Heart Association

RESEARCH EXPERIENCE

PhD Candidate 2015-present
Lab of David Hackam, Institute of Genetics Medicine, Johns Hopkins School of Medicine

- Investigated the utilization of circulating bone marrow-born cells as a possible therapy for intestinal inflammatory diseases
- Worked effectively in a team of twenty multidisciplinary professionals, leading to the first one to graduate among the same year group
- Collected and documented biomedical research data systematically, resulting in successful thesis dissertation and publication in three peer-reviewed journals, including one first-author paper
- Implemented and optimized the animal surgery protocols in compliance with the institutional regulations, training six scientists on a parabiosis surgical procedure
- Collaborated with cross-departmental scientists advising on conducting animal surgeries, resulting in publication in peer-reviewed journals

- Presented scientific data at weekly lab meetings and reported thesis project progress at departmental seminars

Research Assistant 2013- 2014
Lab of Dong-chul Kang, Institute of Life Science, Hallym University

- Investigated the characterization of stomach cancer stem cells and their resistance to chemotherapies
- Developed drug screening protocol for innovative combination of chemotherapies targeting stomach cancer cells
- Managed six human cancer-derived tumor cell lines per safety regulations and ethical guidelines

CLINICAL SETTTING EXPERIENCE

Registered Nurse 2009-2010
Medical Clinic, U.S. Department of State, Kuwait City, Kuwait

- Served as the Post's clinic charge nurse with a high level of independence, providing the community health care services to Mission employees and eligible families
- Implemented medical emergency contingency planning in coordination with the Regional Security Officer for the Post, and conducted on-site visits to ensure compliance with protocols
- Liaised with foreign service providers and arranged medical evacuations through the Regional Medical Officer

Registered Nurse 2005-2006
Rehabilitation Unit and Dementia Unit, Carolina Health Care Center, Hope Mills, NC, USA

- Served as a RN team leader, supervised the clinical practice of nursing staff and collaborated with physicians and multidisciplinary team members
- Planned, coordinated, and managed individualized nursing care and provided health education to the elderly
- Provided rehabilitation and long-term patient care in geriatric, rehabilitation, and psychiatric units

Registered Nurse 2000-2001
Thoracic Surgery Unit and Transplant Surgery Unit, Seoul National University Hospital, Seoul, South Korea

- Worked as a staff nurse providing direct pre- and post-care and education for patients undergoing thoracic surgery and organ transplant
- Liaised with interdisciplinary team and implemented personalized care plan based on the nursing process
- Conducted ongoing patient assessments and performed therapeutic nursing interventions

PUBLICATIONS

Sung, J., Sodhi, C. P., Voltaggio, V., Hackam, D. J. "Circulating cells of bone marrow origin protect against intestinal inflammation." (In preparation)

Hou, S., **Sung, J.**, Hackam, D. J., Cihakova, D. "The intricacies of cardiac networks: cardiac fibroblasts regulate macrophage ontogeny and function during cardiac injury" (In preparation)

Jia, H., Sodhi, C. P., Yamaguchi, Y., Lu, P., Martin, L. Y., Good, M., Zhou, Q., **Sung, J.**, Fulton, W.B., Nino, D.F., Prindle, T. Jr., Ozolek, J.A., Hackam, D. J. "Pulmonary Epithelial Toll-like Receptor 4 Activation leads to Lung Injury in Neonatal Necrotizing Enterocolitis." *Journal of Immunology*. 2016. 197(3), 859–871.

PRESENTATION

Sung, J., Sodhi, C. P., Voltaggio, V., Hackam, D. J. "Circulating cells of bone marrow origin protect against intestinal inflammation." Department of Surgery Research Day. Baltimore, MD, 2018

Sung, J., Sodhi, C. P., Voltaggio, V., Hackam, D. J. "Circulating cells afford protection against radiation-induced intestinal injury in a murine parabiosis system." Johns Hopkins Bayview Research Symposium. Baltimore, MD, 2017

Sung, J., Sodhi, C. P., Voltaggio, V., Hackam, D. J. "Circulating cells afford protection against radiation-induced intestinal injury in a murine parabiosis system." American Society of Human Genetics. Orlando, FL, 2017

Sung, J., Sodhi, C. P., Voltaggio, V., Hackam, D. J. "Circulating cells afford protection against radiation-induced intestinal injury in a murine parabiosis system." Department of Surgery Research Day. Baltimore, MD, 2017

ACADEMIC AWARDS

Chancellor's List, UNC Pembroke	2011-2012
Chancellor Incentive Scholarship, UNC Pembroke	2012

UNCP Friends of the Library Book Scholarship, UNC Pembroke	2012
Biology Faculty Award for Excellence, UNC Pembroke	2011
Dean's List, Ewha Womans University	1997
Honor Scholarship, Ewha Womans University	1997-1998

COMMUNITY AND OUTREACH EXPERIENCE

Womack Army Medical Center 2005
Fort Bragg, NC

- Assisted medical teams at Oncology clinic

Veneratio Vitae Club 1997-1999
Seoul, South Korea

- Worked as a community health student nurse

National University of Mongolia 1997
Ulaanbaatar, Mongolia

- Exchanged Mongolian traditional medical procedures and cultural information

Australian Trust for Conservation 1996
Tasmania, Australia

- Participated in preservation of the Australian environment